Lipoprotein(a), *Chlamydia pneumoniae*, leptin and tissue plasminogen activator as risk markers for valvular aortic stenosis

C.A. Glader*a*, L.S. Birganderb, S. Söderbergb, H.P. Ildgruben*c*, P. Saikkud, A. Waldenströmb, G.H. Dahlén*a

*a* Department of Medical Biosciences, Clinical Chemistry, Umeå University, Sweden

*b* Department of Public Health and Clinical Medicine, Umeå University, Sweden

*c* Department of Surgical and Perioperative Sciences, Umeå University, Sweden

*d* Department of Medical Microbiology, University of Oulu, Finland

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**Aims** The aim of the present study was to identify risk markers for the development of valvular aortic stenosis (AS). Lipoprotein(a) (Lp(a)) and *Chlamydia pneumoniae* IgG antibody titres in plasma and in circulating immune complexes as well as leptin and tissue plasminogen activator (t-PA) in plasma were studied.

**Methods and Results** One hundred and one patients (41 women and 60 men, mean age 71±8 years) with significant AS and 101 age- and sex-matched controls were included in this study. All patients underwent aortic valve replacement at the University Hospital in Umeå, Sweden. The controls had no symptoms of cardiovascular disease and they were examined echocardiographically. An Lp(a) level ≥480 mg l⁻¹, *C. pneumoniae*-specific IgG titre ≥1/128, a high leptin level and a high t-PA mass concentration in plasma were identified as risk markers for AS. A strong synergism between Lp(a) and *C. pneumoniae* IgG antibodies in circulating immune complexes was found.

**Conclusion** Our data indicate that a chronic *C. pneumoniae* infection and a high plasma Lp(a) level might influence and aggravate aortic heart valve sclerosis via the formation of circulating immune complexes. The present study also strongly suggests an association between high plasma leptin, t-PA mass concentration and AS.

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**KEYWORDS** Lipoprotein(a); valvular aortic stenosis; *chlamydia pneumoniae*; circulating immune complexes; leptin; tissue plasminogen activator

**Introduction**

Valvular aortic stenosis (AS) is a disease predominantly found in the elderly, and degenerative valvular AS has structural similarities to atherosclerotic lesions.¹² As the development of this disorder may be an active process mediated by immunological mechanisms³, AS may partly be the end result of lipid accumulation and chronic inflammation, as in other atherosclerotic diseases.

A high lipoprotein(a) (Lp(a)) level has been shown to be an independent risk factor for the development of cardiovascular disease⁴–⁵, but the pathogenic mechanisms have still not been fully explained. In addition to traditional risk factors, an infectious aetiology of atherosclerosis has been proposed and a number of different pathogens have been investigated. The first serologic evidence of an association between the obligate intracellular
bacterium *C. pneumoniae* and atherosclerosis in coronary arteries was provided in 1988. Interactions between Lp(a) and *C. pneumoniae* in the development of atherosclerosis has previously been proposed and partly confirmed in patients with ischemic heart disease. The aim of the present study was to investigate the relevance of these suggested interactive effects in patients with valvular AS.

Other recently discussed mediators of the development and/or aggravation of cardiovascular disease include leptin and tissue plasminogen activator (t-PA). Leptin, the recently discovered adipocyte derived and cytokine related hormone, has been shown to be a risk marker for acute myocardial infarction and stroke. It is related to endothelial and immune mechanisms that could be of importance for the development of atherosclerotic disease. High levels of t-PA antigen in plasma possibly indicates endothelial dysfunction and inflammation and is a known risk marker for myocardial infarction.

**Methods**

One hundred and one patients with sclerotic valvular AS consecutively underwent aortic valve replacement between June 1997 and October 1998 at Umeå University Hospital in northern Sweden. Each patient was interviewed and a questionnaire was completed. Clinical data including height, weight, blood pressure, medication and previous diseases were collected from the patients’ medical records. All patients underwent coronary angiography and were examined echocardiographically prior to operation. The aortic valve area was calculated by use of the equation of continuity. One patient had had an acute myocardial infarction within 3 months of sampling and was excluded because of a possible acute phase reaction.

The control subjects were recruited from the municipal census list of Umeå and each was matched to one patient according to age (±3 months) and sex. The controls were sampled between September and December 1998. They were interviewed regarding their health status and a questionnaire was completed. To fulfil the inclusion criteria, control subjects were not allowed to have a history of rheumatic fever, cardiovascular disease of any kind, diabetes mellitus, rheumatic diseases or malignancies. They were not allowed to be on lipid-lowering drugs. A time latency of 3 months was applied for individuals with a possible acute phase reaction due to recent infections or surgery. Blood pressure was measured in a horizontal position, on two different occasions after a resting period of at least 15 min. Weight and height were measured and body mass index (BMI) was calculated as total body weight (kg)/height squared (m²). All control subjects were examined echocardiographically with an Acuson Sequoia 512. They had no signs of significant valvular AS and their aortic valve area ranged from 1.4 to 3.7 cm². All controls had a normal left ventricular systolic function.

In the conditional logistic regression analyses hypertension was defined as systolic blood pressure ≥160 mmHg and/or diastolic blood pressure ≥95 mmHg and/or being on antihypertensive treatment. Smokers were defined as those reporting daily smoking. Previous smokers were classified as non-smokers.

Blood samples were prepared within 2 h and kept frozen at −80 °C until analysed. All samples were analysed with the identities (whether case or control) masked. Circulating immune complexes were precipitated using polyethylene glycol. The patient sample was added to an equal volume of 7% polyethylene glycol and incubated overnight at +4 °C. After centrifugation at 7500 rpm for 15 min the pellet was washed twice with 3.5% polyethylene glycol borate. The precipitates were then dissolved in phosphate buffered saline to the original volume. The dissolved immune complexes and plasma were measured for the specific apolipoprotein(a) content with a tint enzyme linked immuno sorbent assay (ELISA) (Biopool AB, Umeå, Sweden). Two different threshold values for Lp(a) in plasma have been established, 300 mg/l and 480 mg/l. Both values were tested separately as cut-off points in the statistical analyses.

The associations between Lp(a) in plasma and in circulating immune complexes was found to be linear. The correlation between Lp(a) in plasma and in circulating immune complexes was highly significant (Pearson correlation coefficient, r = 0.94, P<0.001). An Lp(a) level in circulating immune complexes equal to 90 mg/l was graphically estimated to be correlated to an Lp(a) level in plasma of 300 mg/l and was therefore chosen as the cut-off value. The Lp(a) content in circulating immune complexes and in plasma was analysed both as a continuous and a categorical variable.

The contents of *C. pneumoniae*-specific IgG antibodies in the dissolved complexes and in plasma and the *C. pneumoniae*-specific IgA antibodies in plasma were determined by a microimmunofluorescence (MIF) method using TWAR strain Kajaani 6 as antigen. A *C. pneumoniae* IgG level ≥1/2 in circulating immune complexes was defined as
positive. An IgG titre in plasma of ≥1/32 was judged as positive and interpreted as a current or previous C. pneumoniae infection. The C. pneumoniae IgG and IgA measurements and the plasma precipitations were carried out at the National Public Health Institute in Oulu, Finland.

Total cholesterol concentrations in plasma were determined by an enzymatical reflectance spectrophotometry dry chemistry method (the Ektachem Clinical Chemistry Slide (CHOL)) on multianalyser Vitros 950 IRC (Johnson & Johnson, Clinical Diagnostics Inc., NY, U.S.A.). Apolipoprotein A-I and apolipoprotein B concentrations in plasma were measured by use of an immunoturbidimetric method according to the manufacturer's protocol (Hitachii 911, Roche, Basel, Switzerland) using polyclonal antibodies (Dako, Glostrup, Denmark). Sampling was performed in a non-fasting condition, it was therefore not correct to analyse for triglycerides. Consequently, LDL-cholesterol levels could not be calculated. A tint enzyme linked immuno sorbent assay (ELISA) (Biopool AB, Umeå, Sweden) was used for determination of t-PA mass concentrations in plasma.

Leptin was analysed with a double antibody radio-immunoassay (RIA) using rabbit anti-human leptin antibodies. I labelled human leptin as tracer and utilizing human leptin as standard (Linco Res., St Louis, MO, U.S.A.). Intra-batch coefficients of variation (CV) were between 2% and 5%, and interassay CV was 4% for low values (2.1–3.9 ng·ml⁻¹), and 1% for high values (16.4–24.6 ng·ml⁻¹).

Lp(a) in plasma and in circulating immune complexes, leptin and t-PA were asymmetrically distributed. These variables were logarithmically transformed before performing parametric statistics. To test the association between increasing levels of risk markers and valvular AS, we categorised the continuous variables into tertiles by the distribution of the referent values using separate cut-offs for men and women. For assessment of the influence of separate biomedical risk markers, we controlled for BMI, hypertension, total cholesterol, apolipoprotein A-I, apolipoprotein B and present smoking in various models. The number of individuals with missing values was at the most three per variable. In the conditional logistic regression analyses, missing values for continuous variables were replaced by the mean value (symmetric variables) or the median value (skewed variables) or the control group, thus ensuring a conservative result.

SPSS 10.0.5 for Windows was used for continuity corrected chi-square tests, Fisher's exact tests and independent samples t-tests. Logistic regression analysis using the conditional maximum likelihood routine designed for matched analysis, available in STATA version 6.0, was used for calculating odds ratios (OR) and 95% confidence intervals (95% CI). All participants gave informed consent and the study was approved by the Research Ethics Committee of the Medical Faculty, Umeå University.

Results

Sixty men and 41 women with valvular AS were included in this study and their characteristics are presented in Table 1. In 67 out of 101 cases, at least one significant coronary artery stenosis was found at angiography, and coronary bypass grafting was performed in 62 of these patients at the same time as the aortic valve replacement surgery. A left main coronary artery stenosis was present in eight cases. The aortic valve area ranged from 0.3 to 1.3 cm² (mean 0.8±0.2 cm²). A moderate aortic valve regurgitation was found in 11 cases. Twenty-four cases used lipid-lowering agents, and seven cases and seven controls used oestrogen replacement therapy.

The distribution of clinical and laboratory parameters in cases and controls and results from the univariate conditional logistic regression analyses are presented in Table 2. Patients with AS had higher diastolic blood pressure. Apolipoprotein B levels were significantly higher in cases compared to controls and associated with an increased risk of valvular AS. The proportion of individuals with an Lp(a) level of 480 mg·l⁻¹ or more in plasma was
significantly higher in cases than in controls (Table 3). This difference was significant among males but not in females. Taking traditional risk markers into account, an Lp(a) level in plasma of 480 mg \(\text{L}^{-1}\) or more was independently associated with valvular AS.

Both male and female cases had higher circulating leptin and t-PA levels than their control subjects. Furthermore, leptin and t-PA levels corresponding to the highest tertiles in controls were significantly associated with valvular AS in both univariate and multivariate analysis.

The distributions of \textit{C. pneumoniae}-specific IgG and IgA titres among cases and controls are presented in Table 4. The proportion of individuals with an IgG titre of 1/128 or above was higher in cases than in controls. The risk of valvular AS in this group was 3.4 times higher compared to those with a \textit{C. pneumoniae}-specific IgG titre of less than 1/128. This increased risk remained after adjustments (OR 3.5; 95% CI 1.3–9.4). The distribution of IgA titres did not differ between cases and controls.

A positive interaction between high Lp(a) levels in plasma and a positive \textit{C. pneumoniae}-specific IgG titre was present (Table 5). A significantly larger proportion of cases (19%) than controls (8%) had an Lp(a) level of 300 mg \(\text{L}^{-1}\) or more and also a \textit{C. pneumoniae}-specific IgG titre of 1/32 or more \((P<0.03)\). This combination was independently associated with valvular AS. The relative excess risk due to interaction was calculated as \((4.6−0.9)−(1.9−1.0) = 2.8\) indicating positive synergy.22,23

The proportion of individuals with an Lp(a) level of 90 mg \(\text{L}^{-1}\) or more in circulating immune complexes did not differ significantly between cases and controls. A larger proportion of cases compared to controls had circulating immune complexes containing a positive (≥1/2) \textit{C. pneumoniae}-specific IgG titre. Subgroup analyses were performed comparing the proportion of individuals with a high/low Lp(a) level and a high/low \textit{C. pneumoniae}-specific IgG titre in circulating immune complexes. A significant difference between cases and controls was present only in subjects with the combination of an Lp(a) level of 90 mg \(\text{L}^{-1}\) or more and a \textit{C. pneumoniae}-specific IgG titre of 1/2 or more in circulating immune complexes \((P = 0.002)\). Subjects with circulating immune complexes containing a positive \textit{C. pneumoniae}-specific IgG titre were 4.5 times more likely to have valvular AS

| Table 2 Clinical and laboratory parameters in cases and controls. Results from the univariate conditional logistic regression analyses expressing odds ratios and 95% confidence intervals |
|-------------------------------------------------|-----------------|-------|---------|-------|
| **Males/females** | **Cases n = 101** | **Controls n = 101** | **P-value** | **OR** | **CI (95%)** |
| Age, years | 70.9 (8.4) | 71.6 (8.5) | Matched | | |
| BMI, kg \(\text{m}^{-2}\) | 26.4 (3.9) | 25.9 (3.8) | 0.3 | 1.042 | 0.960–1.132 |
| <23.6; 24.7 | 12; 14 | 20; 14 | | 1.0 | |
| 23.6–26.9; 24.7–28.9 | 26; 15 | 20; 14 | | 1.9 | 0.8–4.3 |
| 26.9+; 28.9+ | 20; 12 | 20; 13 | | 1.4 | 0.6–2.9 |
| Present smokers | 6 | 13 | 0.1 | 0.4 | 0.1–1.2 |
| Systolic blood pressure, mmHg | 152.7 (24.8) | 148.9 (20.4) | 0.2 | 1.010 | 0.996–1.025 |
| Diastolic blood pressure, mmHg | 84.6 (11.5) | 81.7 (8.5) | 0.045 | 1.033 | 1.002–1.065 |
| Total cholesterol, mmol \(\text{L}^{-1}\) | 6.2 (1.2) | 6.0 (1.1) | 0.3 | 1.140 | 0.885–1.470 |
| Apolipoprotein A-I, mmol \(\text{L}^{-1}\) | 1376.6 (249.6) | 1421.1 (202.6) | 0.2 | 0.999 | 0.998–1.000 |
| <1268; <1393 | 28; 17 | 20; 13 | | 1.0 | |
| 1268–1450; 1393–1595 | 18; 13 | 20; 15 | | 0.6 | 0.3–1.2 |
| 1450+; 1595+ | 14, 11 | 20; 13 | | 0.2 | 0.5–2.1 |
| Apolipoprotein B, mmol \(\text{L}^{-1}\) | 1316.2 (308.9) | 1205.5 (232.0) | 0.004 | 1.002 | 1.000–1.003 |
| <1058; <1190 | 12; 17 | 20; 13 | | 1.0 | |
| 1058–1238; 1190–1312 | 14; 1 | 20; 15 | | 0.5 | 0.2–1.1 |
| 1238+; 1312+ | 33; 23 | 20; 13 | | 1.8 | 0.9–3.5 |

Values are mean (SD) or number. For males, tertile limits are to the left, for females to the right. For categorical variables, \(P\)-values were calculated by use of Pearson chi-square analyses or continuity-corrected chi-square analyses. Independent samples t-tests were used for calculation of \(P\)-values for continuous variables. OR = odds ratio; CI = confidence interval.
than individuals with a negative *C. pneumoniae*-specific IgG titre in circulating immune complexes. In contrast, an Lp(a) level of 90 mg . l−1 or more in circulating immune complexes was not associated with an increased risk of valvular AS. The risk of valvular AS was found to be 9.1 times higher in individuals with the presence of both an IgG titre of 1/2 or more and an Lp(a) level of 90 mg . l−1 or more in circulating immune complexes. A positive interaction was present between these variables and the relative excess risk due to interaction was calculated as (12.3−1.6)−(4.0−1.0) = 7.7.22,23

Discussion

An Lp(a) level of 480 mg . l−1 or more, a *C. pneumoniae*-specific IgG titre in plasma of 1/128 or more, a high leptin level and a high t-PA mass concentration in plasma were identified as significant risk markers for sclerotic valvular AS. A positive interaction between *C. pneumoniae*-specific IgG antibodies and Lp(a) in circulation and in circulating immune complexes was present.

AS is a disease predominantly found in the elderly. In tricuspid aortic valves sclerosis progresses with age after the fifth decade.24 The sclerotic change in congenital bicuspid valves is also age-related, but with a more rapid development, beginning from the second decade with calcification observed from the fourth.25 In the present study, 21% of the valves were found to be bicuspid. Since two out of three bicuspid valves never become stenotic, factors other than the congenital abnormality and age must contribute to the development of sclerosis.26

In the present study, 7% of the cases had a history of rheumatic fever. This figure may be over- as well as underestimated since episodes of acute rheumatic fever may be subclinical.27 Many patients were uncertain of whether or not they actually had gone through the disease. Medical records were often incomplete in this aspect. Non-rheumatic aortic valve disease has been shown to produce alterations similar to those present in chronic rheumatic valvulitis.27 The reason for sclerosis in rheumatic valvular disease in cases might

<table>
<thead>
<tr>
<th>Lp(a) in plasma, mg . l−1</th>
<th>Cases</th>
<th>Controls</th>
<th>P-value</th>
<th>OR</th>
<th>CI (95%)</th>
<th>Adjusted OR</th>
<th>CI (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>265.1 (326.7)</td>
<td>180.8 (280.7)</td>
<td>0.3</td>
<td>1.001</td>
<td>1.000–1.002</td>
<td>1.001</td>
<td>1.000–1.002</td>
</tr>
<tr>
<td>Males</td>
<td>263.1 (277.2)</td>
<td>177.6 (333.3)</td>
<td>0.1</td>
<td>1.010</td>
<td>1.000–1.020</td>
<td>1.001</td>
<td>1.000–1.002</td>
</tr>
<tr>
<td>Females</td>
<td>268.0 (391.7)</td>
<td>185.5 (182.1)</td>
<td>0.8</td>
<td>1.000</td>
<td>1.000–1.000</td>
<td>1.001</td>
<td>1.000–1.000</td>
</tr>
<tr>
<td>Lp(a) in plasma ≥300 mg . l−1</td>
<td>All</td>
<td>28/101</td>
<td>19/101</td>
<td>0.2</td>
<td>1.6</td>
<td>0.8–2.9</td>
<td>1.7</td>
</tr>
<tr>
<td>Males</td>
<td>18/60</td>
<td>9/60</td>
<td>0.1</td>
<td>1.000</td>
<td>1.000–1.000</td>
<td>1.001</td>
<td>1.000–1.000</td>
</tr>
<tr>
<td>Females</td>
<td>10/41</td>
<td>10/41</td>
<td>1.0</td>
<td>1.000</td>
<td>1.000–1.000</td>
<td>1.001</td>
<td>1.000–1.000</td>
</tr>
<tr>
<td>Lp(a) in plasma ≥480 mg . l−1</td>
<td>All</td>
<td>21/101</td>
<td>5/101</td>
<td>0.002</td>
<td>5.0</td>
<td>1.7–14.6</td>
<td>3.4</td>
</tr>
<tr>
<td>Males</td>
<td>14/60</td>
<td>3/60</td>
<td>0.01</td>
<td>1.000</td>
<td>1.000–1.000</td>
<td>1.001</td>
<td>1.000–1.000</td>
</tr>
<tr>
<td>Females</td>
<td>7/41</td>
<td>2/41</td>
<td>0.2</td>
<td>1.000</td>
<td>1.000–1.000</td>
<td>1.001</td>
<td>1.000–1.000</td>
</tr>
<tr>
<td>Leptin, ng . ml−1</td>
<td>Males</td>
<td>8.8 (5.2)</td>
<td>6.2 (4.2)</td>
<td>&lt;0.001</td>
<td>1.114</td>
<td>1.019–1.218</td>
<td>1.230</td>
</tr>
<tr>
<td>Females</td>
<td>22.7 (14.5)</td>
<td>16.0 (9.2)</td>
<td>0.01</td>
<td>1.000</td>
<td>1.000–1.117</td>
<td>1.127</td>
<td>0.995–1.276</td>
</tr>
<tr>
<td>&lt;3.7; &lt;10.5</td>
<td>3; 6</td>
<td>24; 13</td>
<td>1.0</td>
<td>1.000</td>
<td>1.000–1.000</td>
<td>1.001</td>
<td>1.000–1.000</td>
</tr>
<tr>
<td>3.7–6.7; 10.5–17.3</td>
<td>22; 13</td>
<td>16; 14</td>
<td>1.0</td>
<td>1.000</td>
<td>1.000–1.000</td>
<td>1.001</td>
<td>1.000–1.000</td>
</tr>
<tr>
<td>6.7+; 17.3+</td>
<td>34; 21</td>
<td>20; 13</td>
<td>&lt;0.001</td>
<td>0.1</td>
<td>7.2</td>
<td>2.6–19.4</td>
<td>40.1</td>
</tr>
<tr>
<td>t-PA mass, µg . l−1</td>
<td>Males</td>
<td>11.0 (4.4)</td>
<td>7.8 (2.5)</td>
<td>&lt;0.001</td>
<td>1.400</td>
<td>1.161–1.688</td>
<td>1.670</td>
</tr>
<tr>
<td>Females</td>
<td>9.8 (3.2)</td>
<td>7.4 (2.6)</td>
<td>&lt;0.001</td>
<td>1.738</td>
<td>1.216–2.486</td>
<td>20.839</td>
<td>0.541–801.982</td>
</tr>
<tr>
<td>&lt;6.5; &lt;5.7</td>
<td>9; 3</td>
<td>24; 14</td>
<td>1.0</td>
<td>1.000</td>
<td>1.000–1.000</td>
<td>1.001</td>
<td>1.000–1.000</td>
</tr>
<tr>
<td>6.5–8.4; 5.7–8.4</td>
<td>9; 15</td>
<td>17; 14</td>
<td>1.0</td>
<td>1.000</td>
<td>1.000–1.000</td>
<td>1.001</td>
<td>1.000–1.000</td>
</tr>
<tr>
<td>8.4+; 8.4+</td>
<td>42; 23</td>
<td>19; 13</td>
<td>&lt;0.001</td>
<td>0.1</td>
<td>10.9</td>
<td>3.7–32.3</td>
<td>12.1</td>
</tr>
</tbody>
</table>

Values are mean (SD) or number. For males, tertile limits are to the left, for females to the right. For categorical variables, P-values were calculated by use of continuity-corrected chi-square analyses or Pearson chi-square analyses. Independent samples t-tests were used for calculation of P-values for continuous variables. OR = odds ratio; CI = confidence interval. Odds ratios and 95% confidence intervals were calculated using conditional logistic regression analyses. Adjustment performed for hypertension, BMI, total cholesterol, apolipoprotein A-I, apolipoprotein B and present smoking.
just as well be due to or aggravated by inflammation and/or circulating immune complexes. Therefore, none of the cases with suspicion of a previous episode of rheumatic fever were excluded from the analyses.

Valvular AS has been reported to be associated with coronary artery disease.\textsuperscript{28–31} The results in the present study are in agreement with these previous findings since significant coronary artery narrowing was observed in 66% of the cases and coronary artery bypass grafting was performed in 61%. Early lesions of degenerative valvular AS have structural similarities to atherosclerotic lesions.\textsuperscript{1,2} These similarities include basement membrane disruption, extensive lipid disposition, involvement of macrophages and activated T-lymphocytes.\textsuperscript{1,3} The development of valvular aortic sclerosis is probably an active process mediated by immunological mechanisms.\textsuperscript{3} Both atherosclerotic disorders and valvular aortic stenosis might be the end result of chronic inflammation.\textsuperscript{1}

The results from previous studies regarding risk markers associated with valvular AS have been inconsistent. In some studies an association between smoking\textsuperscript{32–34}, hypercholesterolaemia\textsuperscript{34–38}, hypertension\textsuperscript{35,36,39}, BMI\textsuperscript{39} and valvular AS has been described. Other studies have failed to show an association.\textsuperscript{39,40} The present study did not show any relation between valvular AS on the one hand and BMI, present smoking, systolic blood pressure, total cholesterol and apolipoprotein A-I on the other. Diastolic blood pressure was significantly higher among cases. However, this result must be interpreted with caution due to methodological weaknesses. Blood pressure was measured in control subjects on two different occasions. In cases, blood pressure values were recorded once, preoperatively from the medical charts.

In the present study, an Lp(a) level $\geq 480$ mg $\cdot$ l$^{-1}$ was significantly associated with the presence of AS. An association between high plasma Lp(a) levels and valvar AS has been described.\textsuperscript{33,40} Moreover, a high Lp(a) level has in many previous studies been shown to be an independent risk factor for the development of atherosclerotic cardiovascular disease.\textsuperscript{4–8} One important discovery was the striking homology between structures in apolipoprotein(a) and plasminogen.\textsuperscript{41} This finding presented a structural basis for in vivo competition of Lp(a) with plasminogen for binding to

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|}
\hline
\textbf{Chlamydia pneumoniae} & & & & \\
Cases $n=101$ & Controls $n=101$ & \textbf{P-value} & \textbf{OR} & \textbf{CI (95\%)} \\
\hline
IgG $<1/32$ & 34 & 45 & 0.1 & 1.8 & 0.9–3.6 \\
$\geq 1/32$ & 67 & 56 & & & \\
$<1/64$ & 63 & 69 & 0.5 & 1.3 & 0.7–2.4 \\
$\geq 1/64$ & 38 & 32 & & & \\
$<1/128$ & 67 & 86 & 0.003 & 3.4 & 1.5–7.4 \textsuperscript{*} \\
$\geq 1/128$ & 34 & 15 & & & \\
$<1/256$ & 97 & 101 & & & \\
$\geq 1/256$ & 4 & 0 & 0.1 & & \\
$<1/512$ & 99 & 101 & 0.5 & & \\
$\geq 1/512$ & 2 & 0 & & & \\
\hline
\textbf{Chlamydia pneumoniae} & & & & \\
Cases $n=101$ & Controls $n=101$ & \textbf{P-value} & \textbf{OR} & \textbf{CI (95\%)} \\
\hline
IgA $<1/8$ & 11 & 5 & 0.2 & 0.4 & 0.1–1.3 \\
$\geq 1/8$ & 90 & 96 & & & \\
$<1/10$ & 67 & 79 & 0.1 & 1.9 & 1.0–3.8 \\
$\geq 1/10$ & 34 & 22 & & & \\
$<1/20$ & 81 & 85 & 0.6 & 1.4 & 0.6–3.0 \\
$\geq 1/20$ & 20 & 16 & & & \\
$<1/40$ & 83 & 91 & 0.2 & 2.0 & 0.9–4.7 \\
$\geq 1/40$ & 18 & 10 & & & \\
$<1/80$ & 97 & 100 & 0.4 & 4.0 & 0.4–35.8 \\
$\geq 1/80$ & 4 & 1 & & & \\
$<1/160$ & 99 & 100 & 0.1 & & \\
$\geq 1/160$ & 2 & 1 & 1.000 & 2.0 & 0.2–22.1 \\
\hline
\end{tabular}
\caption{Comparison of \textit{C. pneumoniae} IgG and IgA levels in cases and controls using various cut-off points}
\end{table}

\textsuperscript{*}OR = 3.5 and CI (95\%) = 1.3–9.4 when adjusted for hypertension, BMI, total cholesterol, apolipoprotein A-I, apolipoprotein B and smoking.
Table 5  The distribution of Lp(a) and C. pneumoniae-specific IgG antibody contents in plasma and in circulating immune complexes in cases and controls. Results from the conditional logistic regression analysis expressing odds ratios and 95% confidence intervals for different Lp(a) and C. pneumoniae-specific IgG antibody contents in plasma and circulating immune complexes.

<table>
<thead>
<tr>
<th>Case/Control</th>
<th>Lp(a) &lt;300 mg . l⁻¹ in plasma and C.p-IgG in plasma &lt;1/32 (I)</th>
<th>Lp(a) &lt;300 mg . l⁻¹ in plasma and C.p-IgG in plasma ≥1/32 (II)</th>
<th>Lp(a) ≥300 mg . l⁻¹ in plasma and C.p-IgG in plasma &lt;1/32 (III)</th>
<th>Lp(a) ≥300 mg . l⁻¹ in plasma and C.p-IgG in plasma ≥1/32 (IV)</th>
<th>Lp(a) in CIC, mg . l⁻¹</th>
<th>Lp(a) in CIC &lt;90 mg . l⁻¹</th>
<th>Lp(a) in CIC ≥90mg . l⁻¹</th>
<th>C.p-IgG in CIC &lt;1/2</th>
<th>C.p-IgG in CIC ≥1/2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases n = 101</td>
<td>25</td>
<td>48</td>
<td>9</td>
<td>19</td>
<td>80.1 (87.7)</td>
<td>67</td>
<td>34</td>
<td>64</td>
<td>37</td>
</tr>
<tr>
<td>Controls n = 101</td>
<td>34</td>
<td>48</td>
<td>11</td>
<td>8</td>
<td>63.6 (92.5)</td>
<td>77</td>
<td>24</td>
<td>85</td>
<td>16</td>
</tr>
<tr>
<td>P-value</td>
<td>1.0</td>
<td>1.5</td>
<td>1.1</td>
<td>0.1*</td>
<td>0.2b</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.001</td>
</tr>
<tr>
<td>OR</td>
<td>1.0</td>
<td>1.5</td>
<td>1.1</td>
<td>3.1</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>4.5</td>
</tr>
<tr>
<td>CI (95%)</td>
<td>0.7–3.4</td>
<td>0.7–5.3</td>
<td>0.4–3.0</td>
<td>1.2–8.1</td>
<td>1.0–1.01</td>
<td>1.0–1.01</td>
<td>1.0–1.01</td>
<td>1.0–1.01</td>
<td>1.9–10.9</td>
</tr>
<tr>
<td>Adjusted</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CI (95%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C.p = Chlamydia pneumoniae. CIC = Circulating immune complexes. Values are number or mean (SD). P-values were calculated by use of continuity corrected chi-square analyses or Pearson chi-square analyses. Independent samples t-tests were used for calculation of P-values for continuous variables. When comparison made only between group I and II P-value = 0.066, between group I and III P-value = 0.778 and between group I and IV P-value = 0.002. OR = odds ratio; CI = confidence interval. Odds ratios and 95% confidence intervals were calculated using conditional logistic regression analyses. Adjustment performed for hypertension, BMI, total cholesterol, apolipoprotein A-I, apolipoprotein B and present smoking.

*P-value obtained comparing groups I II, III and IV. When comparison made only between groups I and IV P-value = 0.029.

bSignificant difference in men when analysed separately (P-value = 0.042).

cSignificant difference in men when analysed separately (P-value = 0.012).

dP-value obtained comparing groups I II, III and IV.
plasminogen receptors on endothelial cells and to fibrin. This could result in damage to the endothelial cells or inhibition of fibrinolysis on the arterial wall, since Lp(a) resists activation by t-PA. It was therefore highly interesting to estimate the relevance of t-PA in this context in our study.

t-PA levels were significantly higher in cases compared to controls and t-PA levels corresponding to the highest tertile in controls were associated with an increased risk of valvular AS after adjustment for other risk markers. High levels of circulating t-PA antigen is a known risk marker for myocardial infarction. Increased concentrations of t-PA antigen probably indicates reduced fibrinolytic activity since it is a reflection of inactive circulating complexes with plasminogen activator inhibitor-1 (PAI-1). Furthermore, previous findings have suggested a relation between t-PA mass concentration, inflammation and endothelial cell damage.

In addition to traditional risk factors, an infectious aetiology of atherosclerosis has been proposed. A possible relation between *C. pneumoniae* and atherosclerosis in aortic heart valves has been addressed in some previous studies, although one study has failed to demonstrate such an association. However, comparison of results from different studies is difficult since the *C. pneumoniae* polymerase chain reaction (PCR) is a nonstandardized method. In the present study, the prevalence of positive *C. pneumoniae* IgA titres did not differ between cases and controls. Since many individuals never produce IgA antibodies this titre should perhaps not be used as a marker for either acute or chronic *C. pneumoniae* infection. An IgG level in plasma ≥1/128 was related to an increased risk of AS. This result indicates a possible influence of persistent *C. pneumoniae* infection in AS. The MIF test is a sensitive and specific technique for detection of previous *C. pneumoniae* infection, but the results must be interpreted cautiously. It is uncertain whether the presence or the level of *C. pneumoniae* IgG antibody titres by MIF reflects reinfection or a persistent infection. Also, serology has been shown to be an unsatisfactory marker of the *C. pneumoniae*-associated arterial disease status. The bacterium has even been found to a larger extent in tissues from individuals with a low *C. pneumoniae* specific IgG titre than in individuals with a high IgG titre. Besides in immune complexes, antibodies can also be bound in tissues and thereby become non-detectable in the circulation. Detection of *C. pneumoniae* DNA in peripheral blood mononuclear cells may be a better tool than serology for identifying subjects with a persistent *C. pneumoniae* infection.

An hypothesis linking immunological mechanisms, high Lp(a) levels, *C. pneumoniae* and atherosclerosis has previously been described and partly confirmed in patients with acute myocardial infarction. Certain HLA class II DR genotypes have been found to be significantly more common in patients with early coronary artery disease than in controls, especially in patients with high Lp(a) levels. These results indicate that an immune response to Lp(a) antigenic sites might occur, restricted to certain HLA class II genotypes. T cells cannot be activated by an HLA-presented antigen alone. Therefore, secondary simultaneous co-stimulatory signals are needed. Furthermore, the B7 surface molecules expressed by antigen presenting cells are strong co-stimulatory signal carriers. These molecules can also be expressed by macrophages separately from the cell that presents the HLA antigen complex. Macrophages express B7 molecules when they are infected. *C. pneumoniae* is capable of multiplying in monocytes-macrophages and has moreover been found in macrophages in atherosclerotic lesions. Lp(a) is known to be taken up by macrophages. If *C. pneumoniae* is present in macrophages in the heart valve and epitopes from apo(a) are presented at the same time, the T-cells may be activated resulting in T-cell proliferation and release of interferon-γ. This process would result in further activation of macrophages, induction of HLA class II antigen, and the formation of circulating immune complexes containing apo(a) immunoreactive epitopes. The harmful effects of a high Lp(a) level and the presence of a chronic *C. pneumoniae* infection could lead to the formation of circulating immune complexes. These complexes could then attract and bind complement factors thus initiating the inflammatory cascade, possibly causing sclerosis and in time stenosis in the aortic heart valve. Circulating immune complexes may play an important pathogenetic role in various heart diseases. Patients with different types of heart disease have been shown to have higher levels of IgG and IgM containing immune complexes in their serum compared to controls. The relevance of these suggested interactive effects between high Lp(a) levels and positive *C. pneumoniae* IgG titres in patients with AS have previously not been tested. In the present study, a significantly larger proportion of cases than controls were found to have circulating immune complexes containing *C. pneumoniae*-specific IgG antibodies and also a high amount of Lp(a). The presence of circulating immune
independently associated with the development of high levels of leptin and circulating t-PA are the findings in the present study also show that resulting in chronic inflammation. Furthermore, the formation of circulating immune complexes possibly by and a high plasma Lp(a) level might influence the sclerotic process in aortic heart valves by the formation of circulating immune complexes resulting in chronic inflammation. However, more reliable markers of chronic C. pneumoniae infection are needed.

The present study is the first investigating a possible association between high plasma levels of leptin and valvular AS in the elderly. High levels of leptin are related to features of the insulin resistance syndrome\textsuperscript{63}, i.e. central obesity\textsuperscript{65}, hypertension\textsuperscript{67}, dyslipidaemia\textsuperscript{68}, a reduced fibrinolytic capacity\textsuperscript{69} and hyperinsulinaemia.\textsuperscript{66} We have previously identified leptin as a possible risk marker for acute myocardial infarction\textsuperscript{12} and stroke.\textsuperscript{13} Leptin could influence the risk of atherosclerosis through endothelial effects\textsuperscript{14}, promotion of platelet aggregation\textsuperscript{70}, and by activation of the sympathetic nervous system via central mechanisms\textsuperscript{71} which provides a possible link between obesity and hypertension. There are also structural resemblances between cytokines and leptin.\textsuperscript{72,73} Furthermore, leptin enhances a Th1 reaction in lymphocytes\textsuperscript{15} and phagocytosis and cytokine production in macrophages.\textsuperscript{74} Hence, hyperleptinaemia may accelerate atherosclerosis and possibly also valvular aortic sclerosis through its proinflammatory effects.

In conclusion, a chronic C. pneumoniae infection and a high plasma Lp(a) level might influence the sclerotic process in aortic heart valves possibly by the formation of circulating immune complexes resulting in chronic inflammation. Furthermore, the findings in the present study also show that high levels of leptin and circulating t-PA are independently associated with the development of significant stenosis in aortic heart valves.

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