Association of Serum Antibodies to Heat-Shock Protein 65 With Coronary Calcification Levels

Suggestion of Pathogen-Triggered Autoimmunity in Early Atherosclerosis

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Background—Previous studies demonstrated an association between antibodies to mycobacterial heat-shock protein 65 (mHSP65) and carotid artery thickening. We examined whether mHSP65 antibodies are associated with levels of coronary calcification that appear to reflect preclinical coronary artery disease (CAD).

Methods and Results—Serum specimens from 201 healthy asymptomatic subjects (52% male; mean age, 56.6 years) undergoing electron-beam computed tomographic imaging were used to measure levels of mHSP65 and human HSP60 antibodies and antibodies to several infectious pathogens. We found that 84% of the study subjects had anti-mHSP65 IgG antibodies. Mean titers of mHSP65 antibodies were higher (1:394 versus 1:267, \( P = 0.012 \)) in individuals with than in those without elevated levels of coronary calcium (calcium score \( \geq 150 \)). Increasing titers of mHSP65 antibodies were significantly associated, in a dose-response manner, with elevated levels of coronary calcification. Individuals with the highest titers of mHSP65 antibodies (\( \geq 1:800 \)) had an adjusted odds ratio (OR) of 14.3 for having elevated coronary calcium (\( P = 0.004 \)). Association of mHSP65 antibodies with elevated coronary calcification levels was independent of CAD risk factors after multivariate adjustment (\( P = 0.037 \)). Interestingly, mHSP65 antibody titers were correlated with Helicobacter pylori infection (\( P = 0.004 \)), which maintained significance after adjustment for CAD risk factors and seropositivities to other pathogens (adjusted OR, 3.1; 95% CI, 1.4 to 6.6). No association was found between antibodies to human HSP60 and levels of coronary calcification.

Conclusions—Antibodies to mHSP65 are associated with elevated levels of coronary calcification and correlated with \( H. pylori \) infection, suggesting that pathogen-triggered autoimmunity plays a role in early atherosclerosis. (Circulation. 2004;109:36-41.)

Key Words: proteins | infection | calcification
The triggers for the postulated autoimmune responses involving HSPs and atherogenesis are primarily conjectural. Infection, however, appears to be a leading candidate. Bacteria express HSPs that are homologous to human HSPs, and viruses, although not expressing HSPs, incorporate host HSP into their membranes as they leave the host cell in the course of their infectious cycles. It has thus been postulated that infection induces antibodies that are targeted to pathogen-derived HSPs but that cross-react with human HSP. When human HSPs are overexpressed and are present on the surface of endothelial cells, immunopathological processes inducing atherogenic changes in the vessel wall may occur. The results of several studies have been compatible with this concept. For example, an association has been demonstrated between antibodies to mycobacterial HSP65 (mHSP65) and carotid artery thickening.5–7

On the basis of this reasoning, we hypothesized that infection triggers early coronary vascular changes in the host that predispose to the development of CAD and that these changes are caused, at least in part, by antibodies targeted to pathogen HSP but cross-reacting with human HSP60 presented on cells of the vascular wall. In testing the validity of the hypothesis, we identified a group of healthy asymptomatic individuals who were undergoing screening for CAD by electron-beam computed tomographic imaging (EBCT) of the coronary arteries and examined whether an association exists between coronary calcification levels and the presence of antibodies to pathogen-derived HSPs (for which we used a standard pathogen HSP, mHSP65) and to human HSP60.

Methods

Subjects
Under a George Washington University IRB–approved protocol, 201 healthy asymptomatic individuals entered the study. They were scheduled for an EBCT study at George Washington University. At the time of the EBCT scanning, participants were asked to consent and to have blood drawn.

Clinical Examination and EBCT

All participants underwent a clinical examination and completed questionnaires on current and past exposure to traditional CAD risk factors. EBCT provided a quantitative measure of coronary calcification. For the scans, ECG leads were placed, and the patient was positioned supine in the gantry. A preview scan was obtained for positioning the heart. The coronary artery scan-acquisition protocol for the Imatron EBCT scanner consisted of 30 to 32 levels from the aortic valve to the ventricular apex, with each level 3 mm thick. Each level was triggered by the subject’s ECG in end diastole (80% of the RR interval). Coronary calcification was measured by use of the Base Value Region of Interest software, and regions of interest were confirmed manually. Scores were reported using the Agaston scoring system. Calcium score was the prospectively defined cutoff value for defining a level of coronary calcification as low (0–10), medium (11–149), or high (≥150).

Infectious Serology

Serum samples were frozen at −80°C. Serum IgG antibodies were performed with ELISA kits: cytomegalovirus, herpes simplex virus type 1 and type 2 (Wampole), Helicobacter pylori (Meridian Diagnostics), Chlamydia pneumoniae (Savyon Diagnostics), and hepatitis A virus (ETI-AB-HAVK, DiaSorin Inc). Serology values identified as positive were determined according to the manufacturer’s instructions.

Serum Anti-HSP65 and Anti-HSP60 Antibodies

IgG antibodies against mHSP65 or human HSP60 were determined by ELISA. In brief, 96-well microtiter plates were coated with 5 μg/mL recombinant mHSP65 or human HSP60 (StressGen Biotechnologies Corp) in 100 μL carbonate buffer (pH 9.6) per well at 4°C overnight. After washing and blocking with 3% BSA in PBS at room temperature for 3 hours, plates were incubated with 100-μL serum samples diluted in serum diluent (Wampole) to 1 in 50 at room temperature for 1 hour. The plates were incubated with horseradish peroxidase–conjugated goat anti-human IgG diluted 1 in 10 000 with PBS after a further wash, and 100 μL chromogen/substrate solution containing tetramethylbenzidine (Wampole) was then added to wells. Absorbance at 450 nm was measured after 10 minutes after addition of the stopping solution (Wampole). After correction for background absorbance, a serum sample was considered positive for antibodies against mHSP65 or human HSP60 if the optical density exceeded a prospectively defined cutoff value. This cutoff value was calculated from the negative and positive control absorbance values. The positive sample was further diluted to 1:200, 1:400, and 1:800. The OD value assigned to the antibody titer was that which was read from the 1:50 dilution.

Serum CRP Levels

Serum C-reactive protein (CRP) was measured by fluorescence polarization immunoassay technology (TDxFLEx analyzer, Abbott Laboratory). With this assay, 95% of healthy individuals (n = 202) had a CRP level of ≤0.5 mg/dL, and 98% had levels ≤1.0 mg/dL, in their sera. The between-run coefficient of variation of this assay (n = 31) was 4.3% and 2.2% at mean levels of 1.10 mg/dL and 2.94 mg/dL, respectively.

Statistical Analysis

Categorical data were analyzed by the χ2 test or Fisher’s exact test. All tests were 2-sided. The dichotomous variable indicating that the high coronary calcification level (calcium score ≥150) was modeled as a function of other factors was determined by use of multiple logistic regression. The odds ratio (OR) with 95% confidence interval (CI) was used as a measure of likelihood of an elevated coronary calcification level being present in patients with a given risk factor compared with those without that factor, or as a multiplicative factor for each unit increase in age or titers of antibodies to mHSP65 and to human HSP60. The covariates considered were age, male gender, smoking, diabetes, hypercholesterolemia (total cholesterol >240 mg/dL), hypertension (arterial pressure >140/90 mm Hg), family history of CAD, CRP levels, and seropositive status to infections as well as antibodies to mHSP65 and to human HSP60. All covariates were examined as predictors of the elevated coronary calcification levels in univariate analyses and as a group in one multivariate model.

Results

Characteristics of Study Subjects

A total of 201 subjects were studied. Men constituted 52% of the cohort. Their ages ranged from 27 to 79 years (mean, 56.6; median, 56.0 years). Table 1 presents the baseline characteristics of the study subjects in each of the calcium score subgroups. There were 39 individuals (20%) with EBCT evidence of high levels of coronary calcium (calcium score ≥150). As shown in Table 2, the elevated coronary calcium score was independently associated with age and male gender by both univariate and multivariate analysis. The positive association between high coronary calcification and diabetes and hypercholesterolemia were found after adjustment for traditional risk factors.
Anti-mHSP65 and Anti-Human HSP60 Antibodies Versus Coronary Calcification

In our study cohort, 166 of 198 (84%) had anti-mHSP65 IgG antibodies with titers between 1 in 50 and 1 in 800 (mean, 1:292), and 144 of 198 (73%) had anti-human HSP60 antibodies with titers between 1 in 50 and 1 in 800 (mean, 1:229). There was a correction between mHSP65 and human HSP60 antibodies (r = 0.193, P = 0.006). Figure 1 presents the prevalence of anti-mHSP65 and anti-human HSP60 seropositivity in each of the calcium score subgroups. There was an increased prevalence of anti-mHSP65 antibodies, but not anti-HSP60, in the high calcium score group compared with the low calcium score group (P = 0.039).

Further analysis showed that in the anti-mHSP65 seropositive group, 22% of individuals had high coronary calcium scores, whereas in the seronegative group, 6% had elevated scores (P = 0.036). Mean titers of mHSP65 antibodies were higher in individuals with than in those without high coronary calcium (1.394 versus 1.267, P = 0.012). Association of mHSP65 antibodies with high coronary calcification was independent of established CAD risk factors, including age, male gender, smoking, diabetes, hypercholesterolemia, hypertension, family history of CAD, and CRP levels (adjusted OR, 5.53; 95% CI, 1.17 to 26.09). Increasing titers of mHSP65 antibodies were also significantly associated, in a dose-response manner, with elevated levels of coronary calcification after multivariate adjustment (Figure 2). However, no association between either the presence of human HSP60 antibodies or increasing human HSP60 antibody titers and elevated levels of coronary calcification was observed (all P > 0.05, data not shown).

Anti-mHSP65 Antibodies and CAD Risk Factors

Table 3 presents associations between anti-mHSP65 antibodies and CAD risk factors. We found that neither the presence of anti-mHSP65 antibodies nor increasing anti-mHSP65 antibody titers were associated with traditional CAD risk factors (all P > 0.05).

Anti-mHSP65 Antibodies and Infection

Associations of mHSP65 antibodies with infections, including seropositivity to cytomegalovirus, C pneumoniae, H pylori, hepatitis A virus, and herpes simplex virus type 1 and type 2, were analyzed. Interestingly, serum antibody titers to mHSP65 correlated with H pylori infection. The prevalence

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**TABLE 1. Baseline Characteristics of Subjects in Each Calcium Score Subgroup**

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Calcium Score*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low (n=107)</td>
</tr>
<tr>
<td>Age, y (mean±SD)</td>
<td>54.6±9.6</td>
</tr>
<tr>
<td>Male gender, %</td>
<td>40.2</td>
</tr>
<tr>
<td>Smoking, %</td>
<td>34.6</td>
</tr>
<tr>
<td>Diabetes, %</td>
<td>3.7</td>
</tr>
<tr>
<td>↑ Cholesterol, %</td>
<td>55.1</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>28.0</td>
</tr>
<tr>
<td>Family history of CAD, %</td>
<td>53.3</td>
</tr>
<tr>
<td>↑ CRP (&gt;0.5 mg/dL)</td>
<td>13.2</td>
</tr>
</tbody>
</table>

*Calcium score: low (0–10), medium (11–149), and high (≥150).
† P<0.01 vs the low calcium score group.

OR, 5.53; 95% CI, 1.17 to 26.09. Increasing titers of mHSP65 antibodies were also significantly associated, in a dose-response manner, with elevated levels of coronary calcification after multivariate adjustment (Figure 2). However, no association between either the presence of human HSP60 antibodies or increasing human HSP60 antibody titers and elevated levels of coronary calcification was observed (all P > 0.05, data not shown).

**TABLE 2. Association of Presence of High Coronary Calcification With Traditional CAD Risk Factors**

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Univariate P</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, 10 y*</td>
<td>0.001</td>
<td>4.2 (2.3–7.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Male gender</td>
<td>0.013</td>
<td>8.6 (2.9–25.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smoking</td>
<td>0.649</td>
<td>0.6 (0.3–1.6)</td>
<td>0.322</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0.094</td>
<td>7.5 (1.5–37.4)</td>
<td>0.013</td>
</tr>
<tr>
<td>↑ Cholesterol</td>
<td>0.077</td>
<td>2.5 (1.0–6.4)</td>
<td>0.047</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0.264</td>
<td>0.9 (0.4–2.2)</td>
<td>0.852</td>
</tr>
<tr>
<td>Family history of CAD</td>
<td>0.526</td>
<td>1.7 (0.7–3.9)</td>
<td>0.204</td>
</tr>
<tr>
<td>↑ CRP (&gt;0.5 mg/dL)</td>
<td>0.200</td>
<td>0.2 (0.04–1.1)</td>
<td>0.067</td>
</tr>
</tbody>
</table>

*In 10-year increments of age.
†Adjusted covariates: age, male gender, smoking, diabetes, hypercholesterolemia, hypertension, family history of CAD, and elevated CRP levels.

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**Figure 1.** Prevalence of anti-mHSP65 and anti-human HSP60 seropositivity in each calcium score subgroup. Calcium score: low (0–10), medium (11–149), and high (≥150). Ab+ indicates antibody seropositivity. P = 0.039 vs low calcium score group.

**Figure 2.** Adjusted OR for high coronary calcification in various mHSP60 antibody titer groups. When adjusted for traditional CAD risk factors, OR for high coronary calcification increased with increasing titers of anti-mHSP65.
of seropositivity to H. pylori infection was 9.2% in the subgroup with mHSP65 antibody titers of 1:50 to 1:200, 11.1% in the subgroup with antibody titers of 1:400, and 26.3% in the subgroup with antibody titers of 1:800 or more, compared with 6.3% in individuals without mHSP65 antibodies (P for trend = 0.004). A significant association between H. pylori IgG seropositivity and mHSP65 antibody titers remained after adjustment for CAD risk factors (Figure 3). No correlation between either the presence of mHSP65 antibodies or the increase of antibody titers and other infectious agents was found (all P > 0.05, data not shown).

**H. pylori Infection and Coronary Calcification Level**

As shown in Table 4, although H. pylori IgG antibodies were not significantly associated with elevated coronary calcification levels on univariate analysis, the positive association between H. pylori infection and coronary calcification was found on multivariate analysis after adjustment for CAD risk factors and seropositivities to other infectious pathogens (adjusted OR, 3.03; 95% CI, 1.0 to 9.17).

### Discussion

Several lines of evidence suggest that autoimmune mechanisms play a role in atherogenesis, and the important work of Xu et al.\(^5\) and Mayr et al\(^7\) suggests that HSP may be one of the immune targets in this pathophysiological process. Evidence also suggests that infection may contribute to the triggering of these autoimmune responses through antibodies that, although developing in response to infection and targeted to pathogen-derived HSPs, cross-react with highly homologous self-HSPs. Evidence compatible with this includes studies demonstrating that immunization with recombinant mHSP65 induces atherosclerotic lesions in normocholesterolemic rabbits,\(^8\) elevated levels of mHSP65 antibodies are significantly associated with carotid atherosclerosis, and mHSP65 antibodies derived from human serum cross-react with human HSP60.\(^5\)\(^7\) Additional evidence for the biological relevance of pathogen-stimulated antibodies to atherosclerosis derives from the demonstration that antibodies to chlamydial HSP60 isolated from human serum are cytotoxic to heat-stressed human endothelial cells\(^9\) and the recent demonstration by Perschinka et al\(^9\) that antibodies to microbial HSP60/65 recognize specific epitopes on human HSP60 that are present in the arterial wall of healthy young individuals.

Our hypothesis is that infection triggers early coronary vascular changes in the host that predispose to the development of CAD and that these changes are caused, at least in part, by antibodies targeted to pathogen HSP but cross-reacting with human HSP60 presented on cells of the vascular wall. To identify a cohort of individuals with early coronary atherosclerosis, we studied clinically healthy individuals undergoing a screening study for preclinical CAD by using EBCT for the detection of coronary artery calcification. Previous studies have suggested that coronary calcification detected by EBCT is associated with traditional CAD risk factors and predicts preclinical CAD.\(^10\)\(^16\) Compelling evidence appears in the literature that coronary calcification, detected by EBCT, can appear before there is significant coronary luminal narrowing and therefore before clinical evidence of CAD. This fact suggests that coronary calcification is not a “severe endpoint of atherogenesis” but rather begins developing, at least in some people, much earlier than

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**Table 3. Association of Presence and Absence of mHSP65 Antibodies With Traditional CAD Risk Factors**

<table>
<thead>
<tr>
<th>Risk Factor*</th>
<th>Anti-mHSP65 IgG</th>
<th>Seronegative</th>
<th>Seropositive</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>56.9±9.7</td>
<td>56.5±9.6</td>
<td>0.024</td>
<td></td>
</tr>
<tr>
<td>Male gender</td>
<td>46.9</td>
<td>52.4</td>
<td>0.566</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>40.6</td>
<td>36.4</td>
<td>0.648</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>6.3</td>
<td>4.8</td>
<td>0.666</td>
<td></td>
</tr>
<tr>
<td>↑ Cholesterol</td>
<td>50.0</td>
<td>64.2</td>
<td>0.129</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>21.9</td>
<td>32.7</td>
<td>0.224</td>
<td></td>
</tr>
<tr>
<td>Family history of CAD</td>
<td>53.1</td>
<td>57.6</td>
<td>0.642</td>
<td></td>
</tr>
<tr>
<td>↑ CRP (&gt;0.5 mg/dL)</td>
<td>9.4</td>
<td>11.6</td>
<td>1.000</td>
<td></td>
</tr>
</tbody>
</table>

*Data are mean±SD or percentage of patients.
†Univariate analysis.

**Table 4. Association of Coronary Calcification Scores of <150 or ≥150 With IgG Seropositivity to 6 Infectious Pathogens**

<table>
<thead>
<tr>
<th>Antibodies, %</th>
<th>Coronal Calcification Score</th>
<th>&lt;150</th>
<th>≥150</th>
<th>P</th>
<th>Adjusted* P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytomegalovirus</td>
<td>49.1</td>
<td>56.4</td>
<td>0.410</td>
<td>0.968</td>
<td></td>
</tr>
<tr>
<td>Chlamydia pneumoniae</td>
<td>76.6</td>
<td>67.6</td>
<td>0.255</td>
<td>0.494</td>
<td></td>
</tr>
<tr>
<td>Helicobacter pylori</td>
<td>10.7</td>
<td>20.5</td>
<td>0.110</td>
<td>0.050</td>
<td></td>
</tr>
<tr>
<td>Hepatitis A virus</td>
<td>27.2</td>
<td>46.2</td>
<td>0.026</td>
<td>0.333</td>
<td></td>
</tr>
<tr>
<td>Herpes simplex virus type 1</td>
<td>56.0</td>
<td>64.1</td>
<td>0.357</td>
<td>0.713</td>
<td></td>
</tr>
<tr>
<td>Herpes simplex virus type 2</td>
<td>32.7</td>
<td>43.6</td>
<td>0.201</td>
<td>0.254</td>
<td></td>
</tr>
</tbody>
</table>

*Adjusted covariates: age, male gender, smoking, diabetes, hypercholesterolemia, hypertension, family history of CAD, elevated CRP levels, and seropositivities to other infectious pathogens as described in Table 4.
previously suspected. Although the pathogenesis of this early, preclinical calcification is not understood, infection has been suggested to have a role in the development of calcification.17

In the present study, we found that anti-mHSP65 antibodies were significantly increased in the individuals with high levels of coronary calcium (calcium score ≥150). Association of mHSP65 antibodies with elevated coronary calcification levels was independent of established CAD risk factors, and individuals with the highest titer of mHSP65 antibodies (≥1:800) had an adjusted OR of 14.3 for having elevated coronary calcium (P=0.004). However, there was no association of anti-human HSP60 antibodies with elevated levels of coronary calcium. The lack of such an association for anti-human HSP60 antibodies in our asymptomatic cohort might be explained by the fact that autoreactivity to self-HSP60 might develop gradually during disease progression. This would be in line with a recent report by Perschinka et al,9 who identified several human HSP60 epitopes presented in the arterial wall of young healthy individuals and demonstrated the recognition of an increasing epitope number on human HSP60 by bacterial HSP antibodies during the progression of atherosclerosis.

Interestingly, we also found that serum antibody titers to mHSP65 were significantly correlated with \textit{H pylori} infection, which remained after adjustment for CAD risk factors and seropositivity to other infectious pathogens. \textit{H pylori} produces a 58-kDa HSP that has been shown to stimulate a strong immune response in patients with gastritis and those with gastric cancer.16,19 Circulating antibodies to the HSP60 family in patients with \textit{H pylori} infection have been reported.20 Recently, a positive association between \textit{H pylori} infection and elevated levels of mHSP65 antibodies in patients with carotid thickness has been demonstrated by Mayr et al.21 We demonstrated, in addition to the correlation between mHSP65 antibodies and \textit{H pylori} infection, a positive association between antibodies to \textit{H pylori} infection and coronary calcification. Thus, our findings and others suggest that \textit{H pylori} HSP may contribute to atherosclerosis by inducing anti-\textit{H pylori} HSP antibodies that, because of the homology between \textit{H pylori} HSP with the human HSP, cross-react with host HSP and induce immunopathology by molecular mimicry.

One of the limitations of the present study was the lack of measurement of IgA antibodies for \textit{C pneumoniae} infection. Several recent studies have reported that the presence of IgA is a better marker than IgG antibodies for chronic \textit{C pneumoniae} infection, because IgG antibodies to \textit{C pneumoniae} often reflect previous exposure rather than ongoing chronic infection. Mayr et al22 demonstrated that IgA antibodies to \textit{C pneumoniae} were associated with carotid and femoral atherosclerosis and were correlated with antibodies to mycobacterial HSP65. Similarly, Huittinen et al23 found that high levels of \textit{C pneumoniae} IgA but not IgG antibodies were associated with the risk of coronary events and were also associated with human HSP60. In addition, although we found a positive association between antibodies to \textit{H pylori} infection and coronary calcification, there are several important considerations for associations of \textit{H pylori} infection with atherosclerosis. First, certain factors, such as age and low socioeconomic status,24 are related to both \textit{H pylori} infection and the presence of cardiovascular disease. An association of \textit{H pylori} infection with cardiovascular disease could be influenced by these confounding factors. Second, \textit{H pylori} strains bearing the cytotoxin-associated gene A seem to be more decisive for their pathogenic contribution in atherosclerosis, although the mechanisms are unknown.21–25 Moreover, smoking is a stress factor for endothelial cells. Evidence has indicated that both active and passive cigarette smoking is associated with endothelial dysfunction, and endothelial abnormalities may predispose to the atherogenic and thrombotic problems associated with cigarette smoking.26 However, no association of smoking with the levels of coronary calcification and calcium score was found in the present study, suggesting that the mechanisms leading to endothelial dysfunction and to coronary calcification differ.

In conclusion, this study demonstrated that mHSP65 antibodies are associated with elevated levels of coronary calcification in clinically healthy individuals and correlated with \textit{H pylori} infection. This finding is especially interesting when it is considered that antibodies to human HSP60 were not associated with calcifications in this asymptomatic cohort, whereas they are associated with CAD in a symptomatic patient cohort.4 These results suggest that pathogen-triggered autoimmunity deriving from the generation of anti-mHSP65 antibodies cross-reacting to host HSP60 plays a role in early atherosclerosis and that autoreactivity to self-HSP60 might develop gradually during disease progression. Although this study examined associations between coronary calcification, mHSP60 antibodies, and \textit{H pylori} infection, the question of whether anti-HSP immunity has a role in the calcification process, a possible mechanism for elevation of coronary calcification, would be a very worthy subject of future investigations.

References


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