Independent and Joint Effects of Antibodies to Human Heat-Shock Protein 60 and *Chlamydia pneumoniae* Infection in the Development of Coronary Atherosclerosis

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Background—Studies have suggested that the prevalence of antibodies against heat-shock proteins (HSPs), *Chlamydia pneumoniae* (*Cpn*), and cytomegalovirus (CMV) is associated with coronary artery disease (CAD), but the independent or joint effects of human (h) HSP60 antibodies and these pathogens in patients have not been fully elucidated.

Methods and Results—A total of 405 subjects (276 patients with CAD and 129 control individuals) were tested for serum antibodies to hHSP60, *Cpn*, and CMV immediate-early-1 (IE1) antigens. Patients were also assessed for serum cholesterol, triglyceride levels, and smoking habit. Significantly elevated levels of antibodies to hHSP60 and *Cpn* but not to CMV-IE1 antigens were documented in CAD patients. Multiple logistic regression analysis and subanalyses of selected subjects showed that these associations were independent of age, sex, smoking, and serum lipid levels. Antibodies to hHSP60 and *Cpn* did not correlate quantitatively; however, the relative risk of disease development was substantially increased in subjects with high antibody levels to both hHSP60 and *Cpn*, reaching an odds ratio of 82.0 (95% CI 10.6 to 625.0).

Conclusions—High levels of antibodies to hHSP60 and *Cpn* are independent risk factors for coronary atherosclerosis, but their simultaneous presence substantially increases the risk for disease development. **(*Circulation*. 2001;103:1503-1508.)**

Key Words: coronary disease ■ antibodies ■ epidemiology ■ *Chlamydia pneumoniae*

The role of infectious agents in the pathogenesis of atherosclerosis has long been suggested. The pathogens implicated in the development of an inflammatory reaction, the first step in the disease process, include *Chlamydia pneumoniae* (*Cpn*) and cytomegalovirus (CMV).[1,2] An autoimmune component of atherosclerotic disease has also been proposed, and heat-shock protein 60 (HSP60) has been reported as a putative autoimmune antigen.[3]

From 50% to 80% of adults worldwide have antibodies to *Cpn*, a common respiratory pathogen. *Cpn* replicates intracellularly and expresses various proteins, including the major outer membrane protein (MOMP) and chlamydial HSP60. Chronic infection with *Cpn* is common.[4]

CMV is among the infectious agents that usually cause no symptoms in immunocompetent individuals but do establish latent infection. From 50% to ≈100% of adults are seropositive. The immediate-early (IE) proteins of CMV influence transcription from cellular and viral promoters, are involved in escape from immune surveillance and in establishment of virus latency, and are the first proteins expressed after virus reactivation.[5]

Numerous studies have reported increased *Cpn* antibody levels in atherosclerotic patients, although several prospective studies found no significant correlation between the presence of *Cpn* antibodies and incidence of myocardial infarction.[6] The seroepidemiology of CMV infections in atherosclerotic and control individuals is even less clear, with results suggesting elevated CMV antibody levels in patients with primary atherosclerosis[7] or with restenosis after angioplastic surgery,[8] or no association.[9] Human CMV-IE antigens have been implicated in the restenosis process, because IE proteins are locally reactivated in the coronary walls, and the IE2 protein binds to and suppresses the function of tumor-suppressor p53 in the infected vessel wall.[10] No studies to date have included measurement of CMV-IE antibodies in seroepidemiological analyses of atherosclerosis or restenosis development.

Significantly increased levels of serum antibodies to HSP65 have been reported in patients with coronary and

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carotid atherosclerosis.[11–13] Some bacteria contain HSPs, and some viruses induce overproduction of human (h) HSP60 by the infected cell, leading to immunogenicity of the protein due to structural alterations or posttranslational modifications.[14] Thus, antigenic mimicry by HSPs may be the link between infections and atherosclerosis.

Most seroepidemiological studies have focused on measurement of antibodies against a single pathogen or against HSPs. A few studies have assessed antibodies to multiple pathogens, including CMV and *Cpn*,[15–17] or to *Cpn* and HSPs[18] in the same individuals. In the present study, we measured antibody levels against *Cpn*, CMV-IE1, and hHSP60 in the same case/control population to further examine the potential combined effects of these factors in the development of atherosclerotic disease.

Methods

Study Population

The study population of 405 subjects (aged 35 to 77 years) was divided into 4 groups. Group 1 consisted of 248 patients with severe coronary artery disease (CAD) who underwent coronary angiography[19] in 1995 to 1996 at the National Institute of Cardiology, Budapest, Hungary, with significant stenosis (>50% occlusion), clinical signs of stable or unstable angina pectoris, and typical ECG abnormalities. Group 2 comprised 28 patients with mild CAD who underwent angiography at the same institute, with nonsignificant stenosis (<50% occlusion), clinical signs of stable or unstable angina pectoris, and typical ECG abnormalities. Group 3 comprised 53 control patients with clinical symptoms of stable angina pectoris and no coronary alterations detected on angiography. Finally, group 4 was composed of 76 blood donors who were apparently healthy on physical examination and were without history of CAD. All patients in group 1 received aortocoronary bypass graft by open heart surgery. Patients in groups 1, 2, and 3 received either antihypertensive therapy (when systolic/diastolic blood pressure measured in sitting position was >140/90 mm Hg) or lipid-lowering therapy (when serum total cholesterol or triglycerides exceeded 5.6 mmol/L or 2.2 mmol/L, respectively). In some analyses, data from group 1 (severe CAD) and group 2 (mild CAD) were combined (combined group A), and data from group 3 (patient controls) and group 4 (blood donors) were combined (group B). Smoking habit of patients in groups 1, 2, and 3 was recorded. The project was approved by the Ethics Committee of the Semmelweis University, Budapest, Hungary; informed consent forms were signed by the patients.

Cpn Antibodies

Sera were tested at a 1:128 dilution for *Chlamydia*-specific IgG antibodies with a microimmunofluorescence assay (ServiMif *Chlamydia*, SERVIBIO), according to the manufacturer's instructions. Sera were designated as positive (titer ≥1:128) or negative (titer <1:128) based on typical fluorescence associated with evenly distributed Chlamydia organisms. Sera positive for *Chlamydia trachomatis* or *Chlamydia psittaci* antigens were excluded from the study.

CMV-IE1 Antibodies

Serum IgG antibodies to CMV-IE1 antigen were determined by in-house–developed ELISA. For antigen production, chicken embryo fibroblasts were infected with a recombinant canarypox virus expressing CMV-IE exon4 protein.[20] Parental canarypox virus–infected cell lysate served as control antigen. Serum samples were tested in dilution of 1:100 in a standard ELISA. Optical density (OD) values measured on parental canarypox antigen were subtracted from OD values on canarypox CMV-IE exon4 antigen. Antibody levels were considered "low" at a calculated OD <1.00 and "high" at OD≥1.00.

hHSP60 Antibodies

IgG reacting with recombinant hHSP60 (SPP-740, StressGen) was quantified by ELISA at a serum dilution of 1:500 as described previously.[21] Serial dilutions of a control anti-hHSP60 rabbit polyclonal antiserum (StressGen SPA-840) were used as standard. OD values were calculated in arbitrary units per milliliter relative to the standard.

Clinical Laboratory Procedures

Serum cholesterol and triglyceride were measured PAP (peroxidase-anti-peroxidase) by enzymatic colorimetric assay using enzachol-F and ENZGlycid reagents (Diachem). LDL cholesterol was calculated according to the Friedewald formula.[22] The level of lipoprotein(a) [Lp(a)] was determined by ELISA.[23]

Statistical Analyses

All statistical analyses were performed with SPSS for Windows program version 9.0. Differences between groups in continuous variables were estimated with independent-sample *t* test, nonparametric Mann-Whitney *U* test, or Kruskal-Wallis test. For dichoto-

TABLE 1. Clinical Characterization and Risk Factors in Patients With Severe and Mild CAD and Control Subjects

	Patients (Combined Group A) (n=276)	Controls (Combined Group B) (n=129)	*P*
Age, y			
Mean	58.7	56.3	0.042*
Range	35–77	35–77	
Male sex, %	77.2	62.8	0.004*
High level of anti-CMV-IE1†, %	35.5	31.0	0.431
Anti-hHSP60, U/mL			
Median	102.2	49.0	<0.0001*
Range	0–2410	0–722	
High (>160 U/mL) level of anti-hHSP60‡, %	31.5	3.9	<0.0001*
Cpn-positive§, %	79.3	60.5	0.0001*

*Significant difference.
†OD >1.0 at serum dilution 1:100.
‡Highest quartile.
§Titer ≥1:128.

mous variables, χ^2 test or Fisher's exact test was used. ORs and 95% CIs were calculated. All tests were 2-tailed. Logistic regression was used to evaluate potential confounding by covariables and to calculate adjusted ORs. To assess the effect of high hHSP60 antibody levels on CAD development, dichotomous variables were created for hHSP60 antibody levels, ie, high (highest quartile) versus low (lower 3 quartiles of the distribution). This approach was selected because of skewed distributions of hHSP60 antibodies and because the risk associated with hHSP60 antibodies did not differ in the lower quartiles of distribution. Sets of 4 binary indicators were created for each interaction investigated. In the joint-effect analyses, subjects with low levels of both antibodies of interest were used as reference to estimate the relative risk of the other 3 combinations. Differences were considered significant at $P<0.05$.

Results

Demographic Characterization and Risk Factors in Patients With Severe/Mild CAD Versus Control Patients/Blood Donors

Table 1 summarizes the demographic data and the CMV-IE1, hHSP60, and *Cpn* antibody levels in the sera of patients with severe and mild CAD (combined group A) versus patient controls and blood donors (combined group B). Because of a slight dissimilarity in age distribution, a difference of borderline significance in the mean age of the 2 groups was seen. The percentage of males was significantly different. There was no significant difference between CMV-IE1 antibody levels (high or low) in the 2 groups. The most striking differences were found in (1) hHSP60 antibody levels, calculated as either median and range of units (continuous variables) or as percentage of individuals with high levels (highest quartile) of anti-hHSP60, and (2) *Cpn* seropositivity (titer ≥1:128) (Table 1). Logistic regression analysis of hHSP60 antibody levels (high and low) adjusted for sex and age confirmed a significantly higher percentage of subjects with high hHSP60 antibody levels in combined group A than in combined group B ($P=0.0004$; OR 5.4; 95% CI 2.1 to 14.0).

Sera of subjects in the clinically more homogeneous groups 1 and 3 were also compared for total serum cholesterol, HDL, LDL, triglyceride, and Lp(a) levels, and smoking habit was recorded. Table 2 summarizes the results of these measurements, as well as levels of hHSP60, *Cpn*, and CMV-IE1 antibodies. Age, sex, and total and LDL cholesterol levels, as well as CMV-IE1 antibody levels, were similar in both groups. HDL cholesterol was lower and triglyceride and Lp(a) levels were higher in group 1 than in group 3. There was a borderline significant difference in the percentage of smokers. Antibody levels to hHSP60 and *Cpn* remained significantly higher in group 1 than in group 3 (Table 2). When data were adjusted for age, sex, smoking habits, and HDL cholesterol, triglyceride, and Lp(a) levels by logistic regression analysis, anti-hHSP60 levels (high or low, $P=0.0037$, OR 9.8, 95% CI 2.1 to 45.9) and the percentage of *Cpn* seropositives ($P=0.047$, OR 2.4, 95% CI 1.01 to 5.72) were significantly higher in group 1 than group 3. The same parameters were also compared in 35 patients (group 1) and 35 controls (group 3) selected on the basis of same age ±4.5 years, same sex, and similar cholesterol values; CMV-IE1 antibody levels were not significantly different, whereas statistically significant differences were seen for *Cpn* seropositivity ($P=0.009$, OR 4.6, 95% CI 1.5 to 14.7) and for hHSP60 antibodies (high or low: $P=0.012$, OR 3.4, 95% CI 1.0 to 12.5). Furthermore, in group 1, there were 40, 28, and 189 patients undergoing treatment with statin, fibrate, and aspirin, respectively, at the time of blood sampling. No differences in the percentage of *Cpn* seropositives or serum concentrations of hHSP60 antibodies were found between patients with or without treatment (not shown).

TABLE 2. Clinical Characterization and Risk Factors in Patients With Severe CAD (Group 1) Versus Control Patients (Group 3)

	Patients With Severe CAD (n=248)	Control Patients (n=53)	*P*
Age, y			
Mean	58	60	
Range	35–76	38–74	0.184
Male sex, %	76.6	67.9	0.220
Smokers, %	81.1	68.4	0.074
Total cholesterol, mmol/L			
Median	6.20	6.20	
Range	3.40–20.00	4.00–10.1	0.409
HDL cholesterol, mmol/L			
Median	1.27	1.30	
Range	0.67–2.05	1.14–1.37	0.0063*
Triglycerides, mmol/L			
Median	2.00	1.60	
Range	0.3–16.6	0.90–6.30	0.016*
LDL cholesterol, mmol/L			
Median	3.96	4.00	
Range	0.77–8.09	0.80–8.00	0.867
Lp(a), mg/dL			
Median	16.23	6.08	
Range	0.01–136.2	0.01–168.1	0.0002*
High level of anti-CMV-IE1†, %	33.8	32.0	1.00
Anti-hHSP60, U/mL			
Median	101.9	56.6	
Range	0.0–2410.0	0.0–722.1	0.0001*
High (>180 U/mL) level of anti-hHSP60‡, %	29.4	5.7	0.0001*
Cpn-positive§, %	79.4	64.2	0.021*

*Significant difference.
†OD >1.0 at serum dilution of 1:100.
‡Highest quartile.
§Titer ≥1:128.

Lack of Correlation of *Cpn* Seropositivity With hHSP60 Antibody Levels; Correlation With Smoking Habit

Comparison of hHSP60 antibody levels (continuous variable) in *Cpn*-negative and *Cpn*-positive individuals revealed no statistical difference between the 2 groups in the total study population, in either CAD group, or in either control group (Table 3). Moreover, in either CAD group, no statistical difference was seen between *Cpn* seropositives and seronegatives in the percentage of subjects with high-level (highest

TABLE 3. Lack of Correlation Between hHSP60 Antibody Levels and *Cpn* Seropositivity

	Anti-hHSP60, Median (Range) (n), U/mL		
Groups	*Cpn* Negative*	*Cpn* Positive†	*P*
Total study population	73 (0–2410) (108)	83 (0–805) (296)	0.740
Severe CAD	102 (1–2410) (51)	100 (0–805) (197)	0.321
Moderate CAD	110 (43–207) (6)	99 (25–293) (21)	0.705
Patent controls	50 (0–722) (19)	72 (0–177) (34)	0.711
Blood donors	53 (13–213) (32)	44 (0–143) (44)	0.415

*Titer ≤1:128.
†Titer ≥1:128.

quartile) hHSP60 antibodies ($P=0.260$ to 0.869) (not shown). By logistic regression analysis of data for *Cpn* seropositivity adjusted for sex, age, and hHSP60 antibodies (continuous variable), the probability value remained significant in all combinations of case and control groups, and ORs varied between 2.2 and 2.6 (Table 4). No correlation between hHSP60 and HCMV-IE1 antibodies was observed (not shown).

In groups 1, 2, and 3, *Cpn* seropositivity was significantly associated with smoking; 81.1% of smokers but only 68.4% of nonsmokers tested positive for *Cpn* antibodies ($P=0.018$).

Joint Effects of Microbial and hHSP60 Antibodies in the Development of Atherosclerosis

The simultaneous occurrence of high hHSP60 and high microbial antibodies was analyzed in combined group A versus combined group B with respect to the relative risk of CAD (Table 5). In subjects with *Cpn* antibody titers ≥1:128 and with high hHSP60 antibody levels (highest quartile), the risk of CAD was dramatically increased relative to subjects with no or low levels of *Cpn* antibodies and low levels of hHSP60 antibodies (lower 3 quartiles) (nonadjusted OR 83.3; adjusted for age and sex, OR 82.0) (Table 5). ORs for subjects with high hHSP60 antibodies and *Cpn* seropositivity (titer ≥1:128) in group 1 were also high compared with group 3 (adjusted for age and sex, $P=0.0007$, OR 38.3, 95% CI 4.7 to 312.5) (not shown). However, the simultaneous presence of high CMV-IE1 and high hHSP60 antibody levels was not associated with increased risk. The simultaneous presence of high levels of CMV-IE1 and *Cpn* antibodies did not change the ORs (Table 5).

Discussion

In our study, elevated *Cpn* and hHSP60 antibodies but not CMV-IE1 antibodies were significantly associated with a population of angiographically confirmed severe and moderate CAD patients versus patient controls and blood donors and with a population of severe CAD patients versus patient controls. Antibodies to hHSP60 and *Cpn* were present independently in the study population, but a potent joint effect of high levels of hHSP60 and *Cpn* antibodies was observed, indicating that the coincidence of *Cpn* infection and high level of autoantibodies to hHSP60 is a strong risk factor for CAD development.

Cpn antibodies have been shown to be associated with increasing age, male sex, and smoking.[6] In the present study, the *Cpn* antibody data were adjusted for age, sex, serum lipids [including Lp(a)], and smoking by logistic regression, and statistically significant differences and ORs suggest a 2- to 3-fold higher risk for CAD in individuals with elevated *Cpn* antibodies.

Our analysis of high and low *Cpn*-specific antibody titers did not distinguish between subjects with low titers (<1:128) and those never infected but rather may distinguish subjects with frequent, recent, or chronic infections from those with less frequent, less recent, or milder infections. However, misclassification of prior infection based on current high antibody status (≥1:128) was probably equal in the case and control groups. Also, blood donors were not examined by angiography, so that some individuals with asymptomatic CAD may have been incorrectly classified as controls. However, such misclassification would be reflected in a lower OR, making the reported OR a conservative estimate.

Bacterial and viral infections can induce immune reactivity against hHSP60, which may serve as a target for autoimmune reactions. Our results for elevated levels of hHSP60 in CAD patients are in agreement with previous observations for HSP65.[11,12] Our study revealed no quantitative correlation between hHSP60 and *Cpn* antibodies as detected by a microimmunofluorescence assay that is most likely specific for MOMP,[24] and the data suggest that hHSP60 and *Cpn* are independent risk factors in the development of the disease. Circulating hHSP60 antibodies might be maintained at higher levels through different mechanisms (for example, through infection with agents containing homologous HSPs). Other mechanisms, such as hypertension[25] or oxidized LDL,[26]

TABLE 4. Logistic Regression Analysis of *Cpn* Seropositivity* Adjusted for Sex, Age, and Anti-hHSP60†

Groups	No. of Cases/Controls Seropositive for *Cpn*	*P*	OR (95% CI)
Patients with severe/moderate CAD (combined group A) vs control patients/blood donors (combined group B)	276/129	0.0010‡	2.4 (1.4–4.0)
Patients with severe CAD (group 1) vs patient controls (group 3)	248/53	0.025‡	2.2 (1.1–4.3)
Patients with severe CAD (group 1) vs blood donors (group 4)	248/76	0.0056‡	2.6 (1.3–5.0)

*Titer ≥1:128.
†Adjusted for continuous variables of anti-hHSP60 units.
‡Significant difference.

TABLE 5. Joint Effects of hHSP60 Antibodies and Microbial Antibodies on the Relative Risk of CAD in Combined Group A Versus Combined Group B

Parameters*	No. of Cases/Controls	Without Adjustment		Adjusted for Age and Sex	
		P	OR (95% CI)	*P*	OR (95% CI)
Cpn-neg–hHSP60 low	37/47	...	1	...	1
Cpn-neg–hHSP60 high	19/4	0.0028†	5.9 (1.8–18.7)	0.0019†	6.8 (2.0–22.8)
Cpn-pos–hHSP60 low	147/77	0.0006†	2.4 (1.4–4.0)	0.0052†	2.2 (1.2–3.7)
Cpn-pos–hHSP60 high	68/1	<0.0001†	83.3 (11.1–625.0)	<0.0001†	82.0 (10.6–625.0)
CMV-IE1 low–hHSP60 low	117/84	...	1	...	1
CMV-IE1 low–hHSP60 high	59/3	<0.0001†	14.1 (4.3–46.5)	<0.0001†	14.1 (4.2–46.7)
CMV-IE1 high–hHSP60 low	72/38	0.2116	1.3 (0.8–2.2)	0.150	1.4 (0.9–2.4)
CMV-IE1 high–hHSP60 high	25/2	0.0034†	8.9 (2.1–38.9)	0.0047†	8.5 (1.9–37.8)
CMV-IE1 low–*Cpn*-neg	44/38	...	1	...	1
CMV-IE1 low–*Cpn*-pos	129/47	0.002†	2.4 (1.4–4.1)	0.017†	2.0 (1.1–3.7)
CMV-IE1 high–*Cpn*-neg	17/14	0.911	1.0 (0.4–2.4)	0.866	1.1 (0.5–2.5)
CMV-IE1 high–*Cpn*-pos	81/28	0.0033†	2.5 (1.3–4.6)	0.0091†	2.2 (1.1–2.4)

**Cpn*-neg indicates titer <1:128; *Cpn*-pos, titer ≥1:128; hHSP60 high, antibodies in highest quartile; hHSP60 low, antibodies in lower 3 quartiles; CMV-IE1 low, OD <1.0; and CMV-IE1 high, OD ≥1.0 at a dilution of 1:100.

†Significant difference.

might also stimulate HSP60/70 expression and, in turn, increased HSP60/70 antibody levels. The independent presence of hHSP60 and *Cpn* antibodies suggests that hHSP60 antibodies are predominantly induced by mechanisms other than *Cpn* infection. Alternatively, high levels of autoantibodies against hHSP60 may be a stable, genetically determined trait (Amarilla Veres, MD, and Tamas Szamosi, MD, unpublished observations, 2000) that predisposes to CAD by formation of abundant hHSP60–anti-hHSP60 immune complexes[21] and may result in intense in situ complement activation and endothelial cell dysfunction.

A previous study[18] concluded that high-titer antibodies to mycobacterial HSP65 correlated with the presence of antibodies to *Cpn* in human sera obtained from atherosclerotic patients and that the HSP65 antibodies cross-reacted with hHSP60, chlamydial HSP, and *Escherichia coli* Gro-EL in ELISA. Our results contrast with this observation, possibly owing to differences in the study populations used, the evaluation of high or low levels of antibodies to *Cpn*, or the type of HSP antibodies investigated (antibodies to mycobacterial HSP65 or hHSP60). However, a recent report[27] that CAD patients were more likely to have IgG antibodies to *Cpn* than were individuals without CAD but that CAD was not associated with antibody response to chlamydial HSP60 supports our observation. Furthermore, marked differences in the complement-activating ability and epitope specificity were found between HSP65 and hHSP60 antibodies present in CAD patients.[21]

We found no significant difference in the percentage of individuals with high anti-CMV-IE1 levels in patients versus controls. A problem throughout seroepidemiological studies of CMV is the high infection rate of the population worldwide (50% to ≈100%).[5] Based on the detection of antibodies to whole CMV, ≈90% of the adult Hungarian population is seropositive (K. Burian, MD, unpublished observation, 1999). Thus, these results do not exclude the possibility of the involvement of CMV in the development of some forms of CAD.

Previous analyses of the simultaneous presence of elevated *Cpn* and CMV antibodies to whole virion in the same populations suggested an association between *Cpn* infection, but not CMV infection, and CAD.[15,17] Our findings are in accord with those data on elevated *Cpn* seropositivity in patients with CAD, and the results obtained by detection of CMV-IE1 antibodies are also consistent with those findings.

We found that the simultaneous presence of hHSP60 antibodies and *Cpn* antibodies is associated with a striking increase in the risk (OR 38.3 to 82.0) for development of CAD. On the other hand, no joint effects of the simultaneous presence of high hHSP60 and high CMV-IE1 antibodies were revealed, indicating differences between microbial infections in the interaction with hHSP antibodies.

In patients with CAD, a potent joint effect has also been described for the simultaneous presence of C-reactive protein, an acute phase protein used as a marker for inflammation, and antibodies to herpes simplex virus 1 (HSV-1); the OR in subjects with high HSV-1 antibody and high C-reactive protein levels was 25.4 (95% CI 2.9 to 220.3).[17] That finding, together with our results, supports the hypothesis that inflammatory reactions, infections with certain pathogens, and hHSP60 induced by various stimuli in endothelial or other cells act independently, but their simultaneous presence greatly increases the risk for disease development.

In summary, our present data indicate the independent presence of elevated *Cpn* and hHSP60 antibodies in CAD patients and the substantially increased OR for CAD in individuals with the simultaneous presence of *Cpn* antibodies at titers ≥1:128 and high levels of hHSP60 antibodies. These results might provide direction in further studies on the mechanisms underlying coronary artery diseases.

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