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Association of Serum-Soluble Heat Shock Protein 60 With Carotid Atherosclerosis

Clinical Significance Determined in a Follow-Up Study

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Background and Purpose—Previous work has shown that soluble heat shock protein 60 (HSP60; sHSP60), present in circulating blood, is associated with carotid atherosclerosis. In the current evaluation, we tested the hypothesis that sHSP60 levels are associated with the progression of carotid arteriosclerosis, prospectively.

Methods—The association of sHSP60 with early atherogenesis (5-year development and progression of nonstenotic carotid plaques) was investigated as part of the population-based prospective Bruneck Study. The current study focused on the follow-up period between 1995 and 2000 and, thus, included 684 subjects.

Results—sHSP60 levels measured in 1995 and 2000 were highly correlated ($r=0.40$; $P<0.001$), indicating consistency over a 5-year period. Circulating HSP60 levels were significantly correlated with antilipopolsaccharide and anti-HSP60 antibodies. It was also elevated in subjects with chronic infection (top quintile group of HSP60, among subjects with and without chronic infection: 23.8% versus 17.0%; $P=0.003$ after adjustment for age and sex). HSP60 levels were significantly associated with early atherogenesis, both in the entire population (multivariate odds ratio, for a comparison between quintile group V versus I+II: 2.0 [1.2 to 3.5] and the subgroup free of atherosclerosis at the 1995 baseline: 3.8 [1.6 to 8.9]). The risk of early atherogenesis was additionally amplified when high-sHSP60 and chronic infection were present together.

Conclusions—Our study provides the first prospective data confirming an association between high levels of sHSP60 and early carotid atherosclerosis. This possibly indicates an involvement of sHSP60 in activating proinflammatory processes associated with early vessel pathology. (*Stroke*. 2005;36:2571-2576.)

Key Words: heat shock protein ■ atherosclerosis ■ progression ■ follow-up

Atherosclerosis is a multifactorial process characterized by accumulation of lipid-laden macrophages and proliferating smooth muscle cells within the vessel wall.^{1,2} Early atherosclerotic lesions (fatty streaks) are also characterized by a comparative abundance of inflammatory cells, like activated T lymphocytes, mast cells, and macrophages, indicating the involvement of immunoinflammatory processes in its pathogenesis.^{1,3,4} One of the candidate antigen, stimulating recruitment of T cells into the plaque, is increasingly believed to be bacterial heat shock protein (HSP) 65 (HSP65).⁵

The HSP60/65 family of chaperone proteins is a group of evolutionarily conserved proteins with a molecular weight of ≈ 60 kDa, which facilitates the folding of newly synthesized polypeptides in an ATP-dependent manner and plays an important role in maintaining the dynamic stability of the

intracellular proteome.^{6–8} Because of their evolutionary conservation, HSPs show a high degree of structural homology all the way from prokaryotes to humans.^{6–8} Human autoantibodies against HSP60/65 have been shown to be cross-reactive against chlamydial, mycobacterial, and other bacterial HSPs.⁹ HSP60, both human and chlamydial, has also been shown to enhance the production of proinflammatory cytokines, like tumor necrosis factor α and matrix metalloproteinase production in macrophages,^{10,11} and increase expression of interleukin 6, adhesion molecules like E-selectin, and intercellular adhesion molecule 1 in vascular endothelial cells.¹² Elevated levels of soluble HSP60 (sHSP60) have been found in patients with borderline hypertension¹³ and correlate with high-carotid intima-media thickness. In a large population-based study, we¹⁴ have demonstrated previously that levels of

Received June 8, 2005; final revision received September 8, 2005; accepted September 16, 2005.

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Stroke is available at <http://www.strokeaha.org>

DOI: 10.1161/01.STR.0000189632.98944.ab

circulating sHSP60 are associated with the presence and severity of carotid atherosclerosis and that subjects with high sHSP60 experienced faster progression of atherosclerosis in the 5 years before the assessment of sHSP. The correlations were independent of age, gender, and other established risk factors. However, these studies do not permit assessment of the temporal relationship between high sHSP60 levels and progression of arteriosclerosis. In the current evaluation, we reassess the association of sHSP60 with progression of arteriosclerosis during follow-up of the same population. The hypothesis was that sHSP60 levels predict the progression of arteriosclerosis during follow-up.

Methods

Study Population

Population recruitment was performed as part of the Bruneck Study.^{15,16} The survey area was located in the north of Italy (Bolzano Province). Special features of the study design and protocol have been detailed previously.¹⁵⁻¹⁷

Clinical Examination and Laboratory Methods

All of the participants completed standardized questionnaires on current and past exposure to candidate vascular risk factors and underwent a clinical examination with cardiovascular and neurological priority.^{14,18} Vascular risk factors were assessed by standard methods as detailed previously.¹⁴⁻¹⁸ Subjects with chronic infections were identified by extensive clinical and laboratory screening as described previously.¹⁸ Briefly, an extensive screening consisting of 2 consecutive phases identified subjects with chronic infections. The first step involved a detailed self-reported medical and medication history, thorough clinical examination, spirometry, extensive laboratory evaluations including urinary analysis, and a review of the Bruneck Hospital databases and other medical records. If the data were inconclusive, in a second step, individuals were referred for additional optional examinations. For example, bronchitis was defined as chronic when cough with expectoration lasted ≥ 3 months in 2 consecutive years. Urinary tract infections were regarded as recurrent in the case of ≥ 3 documented episodes. Periodontitis was defined by self-report.

sHSP60 Assay

The serum sHSP60 concentrations were analyzed using a slight modification of sandwich ELISA described previously.^{14,19} The two monoclonal antibodies used were II-13 and N-20 (Santa Cruz Biotechnology), each reacting against separate nonoverlapping epitopes of HSP60.

Assays of Antilipopolysaccharide, Antimycobacterial HSP65, Antichlamydia, and Anticytomegalovirus Antibodies

The procedure for determining antilipopolysaccharide antibodies was similar to that described elsewhere.²⁰ Serum anti-HSP65 antibodies were measured using the ELISA technique described previously.² Serum antibodies against *Chlamydia pneumoniae* and cytomegalovirus (CMV) were determined using the following commercially available assays, as per their instructions: SeroCP-IgA (Savyon Diagnostics Ltd) and CMV-IgG ELISA (Medac).

Scanning Protocol and Definition of Ultrasound End Points

The ultrasound protocol involves scanning of the internal (bulbous and distal segments) and common (proximal and distal segments) carotid arteries on either side with a 10-MHz imaging probe and 5-MHz Doppler.^{17,21} Atherosclerotic lesions were identified by 2 ultrasound criteria: (1) wall surface (protrusion into the lumen or roughness of the arterial boundary); and (2) wall texture (echo-

genicity). Accuracy of this procedure has been established previously.¹⁵ Incident atherosclerosis was defined by the occurrence of atherosclerotic lesions in vessel segments initially free of atherosclerosis and progression of nonstenotic lesions by a relative increase in the plaque diameter exceeding twice the measurement error of the method.^{15,17,21} Both processes were combined to a single outcome category called "early atherogenesis."¹⁵ Incident vessel stenosis was assumed whenever the progression criterion was met and a narrowing of the lumen $>40\%$ occurred. This stage of atherogenesis was termed "advanced atherogenesis."¹²⁻¹⁵ The 2 progression categories were highly reproducible (κ coefficients >0.8 ; $n=100$).

Statistical Analysis

Correlations between sHSP60 and other parameters were estimated by Spearman rank correlation coefficients. Strength and type of association between baseline sHSP60 (1995) and various stages of atherogenesis (1995 to 2000) were assessed by logistic regression analysis, with the test procedure based on maximum likelihood estimators. The accuracy of fit of each model was assessed by the test of Hosmer and Lemeshow. As to early atherogenesis (5-year incidence/progression of carotid atherosclerosis), separate equations were fitted in the entire population ($n=684$) and in subjects free of atherosclerosis at the 1995 evaluation. Base models were adjusted for age and gender \pm baseline atherosclerosis. Multiple regression analyses were adjusted for fixed sets of covariates that were assessed in previous analyses of the vascular risk profile of the Bruneck study population.¹⁵ sHSP60 was \log_e transformed and odds ratios (95% CI) were calculated for a 1-SD unit increase in variable levels. In additional analysis, quintile groups of sHSP60 were modeled as a set of indicator variables. We performed a test for linear trend by treating the medians in each category of sHSP60 as a continuous variable.

The current analysis and the manner of risk factor adjustment were prespecified to avoid multiple testing. Calculations were performed using the SPSS 11.5 and BMDP software packages. A 2-sided $P<0.05$ was considered significant.

Results

In 232 (33.9%) study subjects, serum HSP60 was not detectable. In the rest of cohort, the median sHSP60 level was 68 ng/mL, with a range of 0 to 11 000 ng/mL. A total of 37 subjects (5.4%) had sHSP60 concentrations >1000 ng/mL; such high concentrations have been shown to cause maximal activation of macrophages and endothelial cells in vitro.¹⁰⁻¹² sHSP60 levels measured in 1995 and in 2000 were highly correlated ($r=0.40$; $P<0.001$), indicating that an individual sHSP60 level may be a consistent characteristic during a 5-year period.

Of 684 subjects, 330 showed early atherogenesis; that is, they developed new atherosclerotic lesions or showed progression of preexisting nonstenotic plaques. Among the 350 subjects free of carotid atherosclerosis at the 1995 evaluation, 75 developed first atherosclerotic plaques.

Table 1 lists the means and proportions of selected demographic characteristics and risk factors in the study population. Additional details have been presented previously.¹⁴ A large number of epidemiological studies have reported about the association between atherosclerosis and various persistent bacterial and viral infections, including chlamydia and CMV.²²⁻²⁵ We were able to show positive correlations between sHSP60 and antipathogen/anti-HSP antibodies in both the 1995 and 2000 evaluation. In both the 1995 and 2000 evaluation, we were able to show positive correlations of sHSP60 with anti-LPS antibodies ($r=0.37$ and 0.28 ; $P<0.001$ each), anti-HSP65 antibodies ($r=0.21$ and 0.27 ;

TABLE 1. Descriptive Characteristics of the Study Population (n=684)

Variable	Mean±SD or n (%) or Median (IQR)
Age, y	56.0±10.2
Female gender, n (%)	354 (51.8)
Hypertension, n (%)	238 (34.8)
Smoking, n (%)	130 (19.0)
Alcohol consumption, n (%)	348 (50.9)
Chronic infection, n (%)	160 (23.4)
LDL cholesterol, mg/dL	146.0±37.5
HDL cholesterol, mg/dL	58.8±16.1
Ferritin concentration, µg/dL	82.0 (33.1–155.8)
Microalbuminuria, µg/L	9.0 (7.0–16.0)
Hypothyroidosis, n (%)	58 (8.5)

IQR indicates interquartile range; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

$P < 0.001$ each), and anti-HSP60 antibodies [$r = 0.26$ (measured in 2000 only); $P < 0.001$]. Correlations were weak and, in part, inconsistent for antichlamydia IgA ($r = 0.08$ and 0.07 ; $P = 0.035$ and 0.08) and anti-CMV IgG ($r = 0.02$ and 0.09 ; $P = 0.59$ and $P = 0.02$). This has been detailed in Table 2. In addition, high levels of sHSP60 were more common among subjects with chronic infections than in those without (top quintile group of HSP60: 23.8% versus 17.0%, $P = 0.003$ after adjustment for age and sex). No correlation was found to exist between C-reactive protein level and sHSP60.

Log_e-transformed sHSP60 levels were significantly associated with early atherosclerosis both in the entire population and subgroup free of atherosclerosis at 1995 baseline (Table 3). To rule out confounding by other vascular risk factors,

TABLE 3. Association of sHSP60 With Early Atherogenesis in the Carotid Arteries (1995–2000)

Variable	Odds Ratio (95% CI)	P Value
Entire study population (n=684)		
Soluble HSP60*	1.20 (1.00 to 1.45)	0.05
Soluble HSP60†	1.26 (1.04 to 1.54)	0.02
Subjects free of AS at baseline (n=350)		
Soluble HSP60*	1.49 (1.11 to 2.01)	0.008
Soluble HSP60†	1.55 (1.12 to 2.14)	0.002

Odds ratios were calculated for a 1-SD increase in log_e-transformed sHSP60 levels. They were derived from logistic regression analysis. *Adjusted for age, gender, baseline atherosclerosis (AS); †Adjusted for age, gender, baseline atherosclerosis, smoking, hypothyroidosis, hypertension, high-density lipoprotein and low-density lipoprotein, cholesterol, microalbuminuria, ferritin concentration, and alcohol consumption.

multiple logistic regression models were adjusted for age, gender, baseline atherosclerosis, smoking, hypothyroidosis, hypertension, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, microalbuminuria, ferritin, and alcohol consumption (Table 2). The association between sHSP60 and early atherosclerosis progression was similar in both genders and all age groups (data not presented). Figure 1 illustrates the relationship between early atherogenesis and sHSP60 quintile groups (quintile I+II was used as the reference group). Significant associations were obtained in both the base and multivariate models and in the entire population and the subgroup free of atherosclerosis at the 1995 baseline. In the latter group, a gradual increase of risk, across the quintile groups, was found. Presence of chronic infection enhanced the predictive significance of sHSP for early atherogenesis (Figure 2). For example, in the subgroup

TABLE 2. Correlation of sHSP60 With Demographic Variables, Vascular Risk Factors, and Antipathogen Antibodies

Variable	Subjects Without AS 1995 (n=350)		Subjects With AS 1995 (n=334)		All Subjects 1995 (n=684)		All Subjects 2000 (n=684)	
	r*	P	r*	P	r*	P	r*	P
Age	-0.06	0.30	-0.06	0.25	-0.09	0.01	-0.02	0.63
Smoking	-0.08	0.13	-0.03	0.56	-0.05	0.18	-0.07	0.07
LDL	-0.03	0.54	-0.04	0.48	-0.04	0.26	-0.01	0.84
HDL	-0.06	0.25	-0.01	0.85	-0.04	0.31	0.03	0.38
Systolic blood pressure	-0.04	0.43	-0.03	0.60	-0.06	0.11	-0.08	0.05
Diastolic blood pressure	-0.10	0.06	-0.05	0.39	-0.03	0.45	-0.09	0.03
TSH	-0.01	0.81	-0.03	0.61	-0.02	0.69	-0.05	0.18
Microalbuminuria	0.03	0.61	0.02	0.68	0.01	0.81	-0.03	0.39
Ferritin	-0.03	0.52	-0.16	0.003	-0.11	0.003	-0.06	0.11
C-reactive protein	0.07	0.17	-0.01	0.79	0.01	0.81	0.06	0.14
Anti-HSP65 AB	0.16	0.002	0.25	<0.001†	0.21	<0.001†	0.27	<0.001†
Antihuman HSP60 AB	NA		NA		NA		0.26	<0.001†
Anti-LPS AB	0.36	<0.001†	0.36	<0.001†	0.37	<0.001†	0.28	<0.001†
Anti-chlamydial IgG	0.11	0.03	0.07	0.21	0.08	0.04	0.07	0.08
Anti-CMV AB	0.02	0.76	0.06	0.26	0.02	0.59	0.09	0.02

AS indicates atherosclerosis; AB, antibodies; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LPS lipopolysaccharide; TSH, thyroid-stimulating hormone; NA, not available; *Data presented are Spearman rank correlation coefficients; †Correlations remain significant after accounting for the multiple comparisons performed.

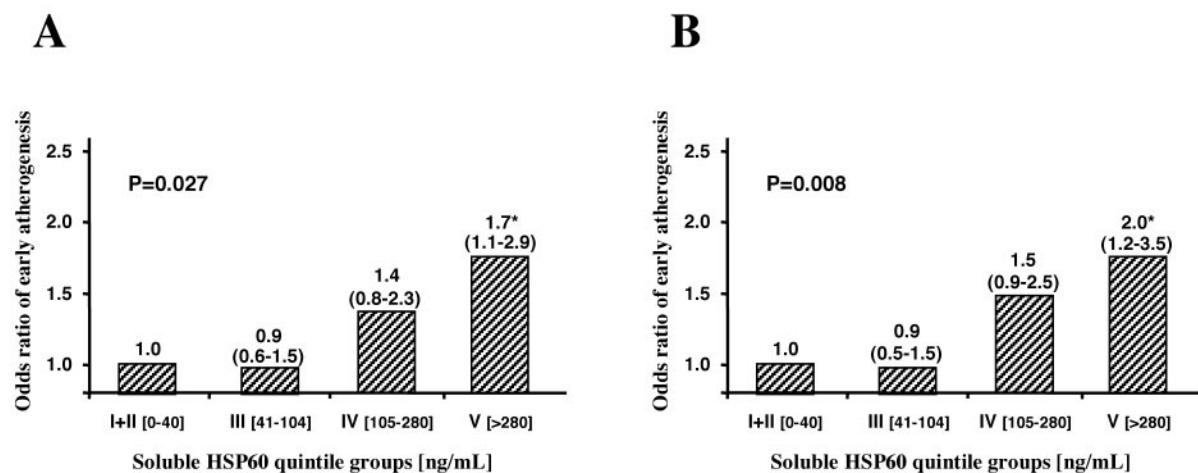
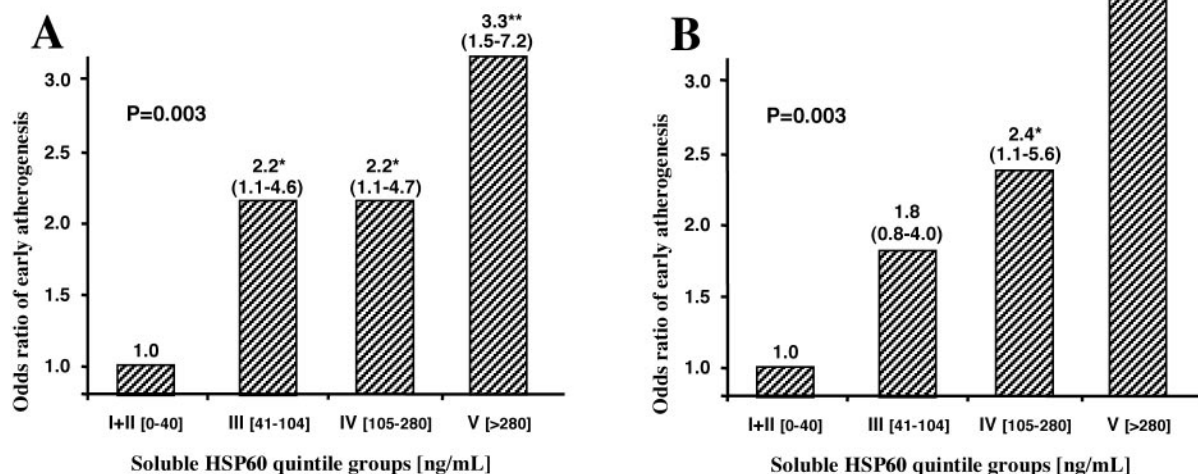
Entire study population (n=684)**Subjects free of AS at baseline (n=350)**

Figure 1. Association of quintile groups for sHSP60 with early atherogenesis in the carotid arteries (1995–2000). Quintiles QI+II (0 to 40 ng/mL) serve as a reference category. Odds ratio (95% CI) for each category is listed above the bar. *P* values are for linear trend. Analysis in A was adjusted for age, gender, and baseline atherosclerosis. Analysis in B was adjusted for age, gender, baseline atherosclerosis, smoking, hypothyroidism, hypertension, high-density lipoprotein and low-density lipoprotein cholesterol, microalbuminuria, ferritin concentration, and alcohol consumption. **P*<0.05; ***P*<0.01.

free of atherosclerosis in 1995, the odds ratio for incident carotid atherosclerosis was 2.0 in subjects with high sHSP60, but this increased to 4.2 when high sHSP60 coexisted with chronic infection (Figure 2A). No association, however, was found to exist between sHSP60 and advanced atherosclerosis [multivariate odds ratio (95%CI) calculated for a 1-SD unit increase in \log_e -transformed sHSP60 0.92 (0.69 to 1.23), *P*=0.59].

Discussion

We have previously shown an association between sHSP60 and severity of atherosclerosis,¹⁴ which has been subsequently confirmed by other groups,^{13,19} but most of these studies were cross-sectional. In this study, we provide the first truly prospective data and demonstrate that subjects with sustained elevation of sHSP60 in their blood are at an

increased risk of early atherogenesis in the carotid arteries (development and progression of nonstenotic atherosclerotic lesions). These findings could be potentially relevant to a better understanding of the role of circulating autoantigens in atherogenesis.

Proteins of the HSP60 family are mostly located in the intracellular compartment (mitochondria and cytosol) and are only expressed on the cell surface under stressful conditions.^{26–28} In this study, we were able to demonstrate a sustained presence of circulating sHSP60 in human blood, over a 5-year observation period. The exact nature and composition of the measured sHSP60 in human serum is yet to be established, but there are a few possibilities. One of the likely sources could be infectious agents, like chlamydia, especially during the lytic phases of their life cycle.²⁹ The association between sHSP60 and antipathogen antibodies and

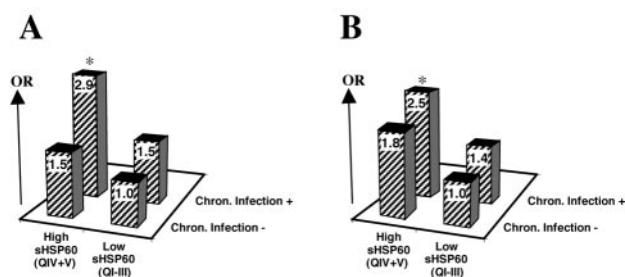
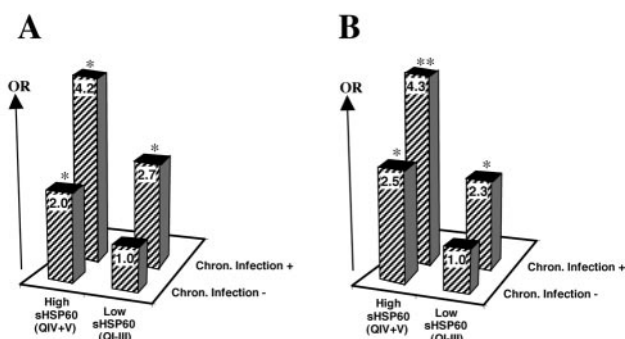
Entire study population (n=684)**Subjects free of AS at baseline (n=350)**

Figure 2. Risk of early atherosclerosis in the carotid arteries (1995–2000) according to baseline sHSP60 level and presence of chronic infection (Chron. Infection). For adjustment see Figure 1. * $P < 0.05$; ** $P < 0.01$.

chronic infection in our study and in other reports¹⁴ and their colocalization in human atheromas¹⁰ lend additional support to this theory. Alternatively, surface-expressed HSP60^{26,30} on stressed cells undergoing apoptosis may be released into the circulation as microparticles. This is corroborated by the finding of circulating microparticles in patients with acute coronary syndrome³¹ and its correlation with the degree of endothelial dysfunction in these patients.³²

In our study, we were able to demonstrate an association between sHSP60 and early but not advanced carotid atherosclerosis. It is interesting that a positive correlation has been demonstrated to exist between peripheral blood T cells reactive to HSP60 and the extent of early vessel pathology as indicated by a high-carotid intima-media thickness in young army recruits.³³ However, a similar association could not be demonstrated in middle-aged and elderly people with advanced atherosclerosis, suggesting that immunity may have a preferential role in early stages of atherosclerosis.

Previous studies have shown that T cells in human atheromas are mostly T-helper 1 cells bearing the α/β receptor;³⁴ however, in the earliest stages of atherosclerosis, there is a relative abundance of T cells bearing the γ/δ receptor.^{34,35} One of the probable antigens recognized by these T cells is HSP, a hypothesis supported by the isolation of T cells from rabbit atheromas⁵ and atherosclerotic plaques obtained from humans,³⁶ specifically responding to HSP60/65. Interleukin 2-expanded T-cell lines derived from atherosclerotic lesions showed a significantly higher HSP60/65 reactivity compared with the cells derived from peripheral blood of the same

donor. Recent work by Zal et al³⁷ demonstrated increased numbers of CD4⁺CD28^{null} T cells in the peripheral blood of patients with acute coronary syndrome in comparison to those with chronic stable angina. These natural killer cells were found to be activated by human HSP60 but not cytomegalovirus, *C. pneumonia*, oxidized low-density lipoprotein, or lipopolysaccharide in a MHC class II-dependent manner. This observation additionally supports the role of HSP60 as an important autoantigen involved in atherosclerosis.

The presence of chronic infection further enhanced the predictive significance of sHSP for early atherosclerosis (Figure 2). sHSP60 is known to mediate adhesion of monocytes to endothelial cells via the CD14 receptor.³⁸ It has also been suggested that HSP60 has a cytokine-like function³⁹ and elicits a release of tumor necrosis factor α from macrophages, causing expression of E-selectin, intercellular adhesion molecule 1, and vascular cell adhesion molecule 1.^{11,12} A total of 5.4% of the subjects in our study had concentrations >1000 ng/mL. Such high concentrations have been demonstrated in various in vitro studies to maximally activate macrophages, endothelial cells,^{11,12} and T cells.³⁷ Similarly, the presence of infections will not only supplement these proinflammatory responses but may also enhance the pool of circulating HSPs^{22–25} and lead to the development of early atherosclerosis.⁴⁰

In summary, we provide the first truly prospective data confirming an association of sustained elevation of sHSP60 levels with early carotid atherosclerosis. This finding is consistent with the involvement of innate and adaptive immunity in atherosclerosis and lends additional support to the role of HSP60 as an important candidate autoantigen.

Acknowledgments

This work was supported by grants from British Heart Foundation (PG/03/132), Oak Foundation (to Q.X.) and the Austrian Science Fund-FWF (project no. P14741; to G.W.).

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