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ORIGINAL PAPER

Extracellular heat shock protein 70 (HSPA1A) and classical vascular risk factors in a general population

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Abstract Atherosclerosis is a chronic inflammatory and autoimmune disease. Candidate molecules/autoantigens include heat shock proteins (HSPs); Hsp70 (HSPA1A) is one of the best studied HSPs. Various studies have shown a correlation between extracellular Hsp70 (eHsp70) and anti-Hsp70/anti-Hsp60 antibody concentration and development of atherosclerosis. A random sample of 456 people aged 40–60 (218 males, 234 females) was studied to investigate the prevalence of traditional vascular risk factors and eHsp70 and anti-Hsp70/anti-Hsp60 antibodies levels, according to the risk of vascular disease. Task Force Chart was applied for classification. Subjects were divided into three groups: G0 (with no vascular risk factor or a risk lower than 5%), $n=239$; G1 (moderated 10–20% risk, who do not have established disease) $n=161$; and G2 (established atherosclerosis disease) $n=52$. eHsp70 and anti-Hsp70 were significantly lower in the atherosclerosis group (group 2) with respect to the other groups. Disease-free people showed the highest anti-Hsp60 concentration compared with the other two groups. A correlation has not been demonstrated between the concentrations of circulating Hsp70 (HSPA1A), anti-Hsp70, and anti-Hsp60 and classical vascular risk factors and C-reactive protein. Low levels of eHsp70 and anti-Hsp70 antibodies should be considered as candidate FRV. Simultaneous decrease of eHsp70 and anti-Hsp70 antibodies would be explained by circulating immune complex formation, and both could be proposed as biomarkers for the progression of atherosclerotic disease. Levels of circulating anti-Hsp60 antibodies may constitute a marker of inflammation in atherosclerosis.

Keywords Anti-HSP antibodies · Atherosclerosis · Hsp60 · Hsp70 · Inflammation · Vascular risk

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Introduction

Cardiovascular disease is the leading cause of death in most western countries (Grau and Marrugat 2008). The inflammatory nature of atherosclerosis was proposed 30 years ago by Ross (Ross and Glomset 1976); his response-to-injury theory (Ross 1999) remains valid to this day. According to this theory, the first step in the development of atherosclerosis is endothelial dysfunction resulting from various stimuli such as hypertension, free radicals produced by tobacco, homocystinemia, the presence of oxidized low-density lipoproteins (LDLs), or infection (Kaperonis et al. 2006). An alteration of the physiologic functions of the endothelium, the onset of inflammatory reactions (with the synthesis of proinflammatory cytokines and other molecules such as C-reactive protein (CRP) on the atheroma plaque), and immune reactions ensue as a result of endothelial dysfunction. Both adaptive and innate immunities are also involved in the etiopathogenesis of atherogenesis (Jara et al. 2006). Specific antigens starting immune response in atherosclerosis include oxidized LDL (Binder et

al. 2004), heat shock proteins (HSPs; Mehta et al. 2005), and β2- glycoprotein (George et al. 2000).

Within the superfamily of HSPs, the proteins most widely studied in relation to the pathogenesis and development of atherosclerosis are Hsp70 (HSPA1A, Kampinga et al. 2009) and Hsp60 (HSPD1). An overexpression of Hsp70 (HSPA1A) in macrophages, smooth muscle cells, and endothelial cells in atherosclerotic plaques in apoE-knockout mice (Kanwar et al. 2001) and the intralesional expression of two genes of the Hsp70 family have been demonstrated (Han et al. 2003). Hsp60 (HSPD1) has been detected in endothelial, smooth muscle, and mononuclear cells in atherosclerotic lesions in humans, but not in blood vessels that do not have these lesions (Xu 2002). The degree of intralesional expression of Hsp60 (HSPD1) correlates positively with the severity of atherosclerosis.

For years, HSPs have been considered exclusively intracellular molecules that could only be released to the extracellular space via a passive mechanism from necrotic cells (Calderwood 2005); currently, however, we know that these molecules may be released by nonnecrotic viable cells, including endothelial cells, through an active mechanism that includes the nonclassic protein release pathway, through which HSP is released both as free protein and within highly immunogenic exosomes (Asea 2008). Furthermore, the presence of Hsp60 (HSPD1) and Hsp70 (HSPA1A) has been shown in the serum of normal individuals (Pockley et al. 1998, 1999; Jin et al. 2004). The role of circulating Hsp70 (HSPA1A) and Hsp60 (HSPD1) in the development of atherosclerosis is not completely defined yet. Increased serum levels of Hsp60 (HSPD1) have been observed in patients with early-stage atherosclerosis that correlate with the degree of carotid artery intima media thickening (Pockley et al. 2000; Xu et al. 2000), which could accelerate the progression of the condition (Xu 2002). In contrast, high levels of circulating Hsp70 (HSPA1A) are associated with low risk of coronary artery disease (Zhu et al. 2003); they appear in hypertensive subjects with a lesser intima media thickening after 4 years of follow-up (Pockey et al. 2003) and in a disease-free population, compared to a group of subjects with metabolic syndrome (Armutcu et al. 2008). Additionally, serum levels of Hsp60 (HSPD1) correlate directly with the classic factors of vascular risk such as total cholesterol and LDL cholesterol (Shamaei-Tousi et al. 2007) and with inflammation markers such as tumor necrosis factor alpha (TNF-α), but not with CRP (Lewthwaite et al. 2002a, b). In turn, Hsp70 (HSPA1A) does not correlate with the concentration of VLDL or triglycerides (Pockley et al. 2000) or with CRP levels (Armutcu et al. 2008). Extracellular Hsp70 (HSPA1A) and Hsp60 (HSPD1) have powerful immune properties: they activate the classic complement pathway, and they participate in the processing and presentation of exogenous antigens and show immune reactivity to endogenous Hsps (Pockley et al. 2008; Molvarec et al. 2009).

Given the potential role of heat shock proteins in the pathogenesis of atherosclerosis, the purpose of this study was to determine the levels of circulating Hsp70 (HSPA1A) and anti-Hsp60 and anti-Hsp70 antibodies in the general population and its relation to the prevalence of vascular risk factors or the presence of atherosclerotic disease.

Material and methods

Study population and design

This was an observational, cross-sectional, epidemiological study on the incidence of vascular risk factors carried out from January 2004 to June 2009. Inclusion criteria include randomly selected voluntary subjects of both sexes aged 40–60 years and employees of the Hospital General Universitario Gregorio Marañón (HGUGM) who signed the informed consent. The study was approved by the Research Committee and the Clinical Research Ethics Committee of the center. All participants provided a clinical history and answered an epidemiological survey including age, personal and family medical history, smoking (number of cigarettes per year and smoking duration; if former smokers, number of years elapsed since smoking cessation), alcohol intake (if yes, grams of alcohol daily), treatments, and occurrence or presence of disease of atherosclerotic etiology. Blood pressure (BP) of all participants was measured with an automated BP recording device after an individual had been sitting quietly for 5 min. Blood was taken for the appropriate laboratory measurements. Exclusion criteria included pregnancy or breast feeding, any systemic infection in the past 3 months, current oncological disease or radiotherapy/chemotherapy, autoimmune disease (rheumatoid arthritis, systemic lupus erythematosus, and sarcoidosis), endocrine disorders (except for diabetes), liver disease, renal failure, glomerulonephritis, congenital heart disorder, oncohematological disease, or allergic disorders.

The Epidemiology and Preventive Medicine Service of HGUGM calculated the sample size. In order to determine whether there is a correlation between serum levels of Hsp70 (HSPA1A) and the development or presence of atherosclerotic disease and to estimate the size of a cohort study, the following premises were presumed: (1) An estimated incidence of the disease of 12.5% in the group with a high serum concentration of Hsp70 (HSPA1A); (2) this population has half the risk of the population with a low concentration of developing the disease (RR 0.5), error protection: alpha 0.05 (bilateral), beta 0.20.

Assessment of vascular risk and classification of subjects

The absolute risk for developing cardiovascular disease in a given period of time (usually 10 years) is estimated based on the presence of prior coronary heart disease (CHD) and the joint assessment of risk factors present (Grundy et al. 1999). A risk factor is defined as a measurable characteristic that is causally associated with increased disease frequency and that is a significant independent predictor of an increased risk of presenting with the disease (Dawber et al. 1951). The calculation of risk in asymptomatic individuals is based on the presence of risk factors. For this purpose, there are several tables based on the follow-up of the Framingham study population (D'Agostino et al. 2001). One of the most widely used is the Task Force's Coronary Risk Chart (Wood et al. 1998). The index of coronary risk of the subjects was obtained considering the presence or absence and the severity of each of the conventional risk factors, taking into account gender, age, smoking status, lifestyle, systolic blood pressure, and total cholesterol. Applying the coronary risk chart, the subjects in this study were classified in three groups: group 0 (G0), subjects with no vascular risk factor or a risk lower than 5%; group 1 (G1), subjects with moderate vascular risk (10–20%) who do not have established disease; and group 2 (G2), subjects with established atherosclerotic disease (Adult Treatment Panel III 2001).

Definition of vascular risk factors (Wood et al. 1998; Graham et al. 2007)

Obesity

Obesity was defined as a body mass index (BMI: kg/m^2) >30 and overweight when the BMI was >25.

Blood pressure

BP of all participants was measured with an automated BP recording device after an individual had been sitting quietly for 5 min. Hypertension has been defined as a systolic blood pressure of ≥140 mmHg and/or a diastolic BP of ≥90 mmHg in at least two measurements taken separately.

Blood lipids

Hypercholesterolemia was defined as a fasting total cholesterol level of ≥200 mg/dl, or LDL cholesterol of ≥130 mg/dl. By hypertriglyceridemia, we understand a condition in which a fasting total triglyceride level is ≥150 mg/dl.

Blood glucose

For epidemiological studies, estimates of diabetes prevalence or incidence should be based on a fasting blood glucose of ≥126 mg/dl (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 1997).

Laboratory tests

Venous blood was drawn after a 12-h fast and centrifuged, and serum samples were frozen at −70°C for subsequent testing. In order to assess causal and conditional vascular risk factors (Lahoz and Mostaza 2007), total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, and glucose were quantified in a Hitachi Modular Analytic SVA autoanalyzer, Roche Diagnostics S.L., Barcelona, Spain.

C-reactive protein

CRP was quantified using a commercial enzyme-linked immunosorbent assay (ELISA; CRP MTPL EIA, DRG Instruments GMBH, Marburg, Germany) according to manufacturer instructions. Final absorbances were measured at 450 nm (Bio Rad 3550 Microplate Reader, Bio Rad Laboratories, Hercules, CA, USA). Values under 5 μg/ml were considered normal. CRP concentrations in the samples were calculated by interpolation in the standard curve obtained (range 1.62-25 μg/mL).

Plasma total homocysteine

Plasma total homocysteine (tHcy) was measured by immunoanalysis of polarization of the fluorescence. Hyperhomocysteinemia was defined as a fasting plasma tHcy concentration >11 μmol/l in women and >15 μmol/l in men (P_{95}).

Serum Hsp70 (HSPA1A) and Hsp60 (HSPD1)

Hsp70 (HSPA1A) and Hsp60 (HSPD1) were quantified in diluted serum 1:5 using the Hsp70 and Hsp60 ELISA kits (EKS-715, EKS-600 Assay-Designs-Stressgen, Ann Arbor, MI, USA) in accordance with manufacturers' instructions. EKS-715 test recognizes recombinant and native Hsp70 (HSPA1A), and there is no cross-reactivity with constitutive Hsp70 (HSPA8) or human Hsp60 (HSPD1). Results were expressed in nanogram per milliliter. The working range (linearity) for Hsp70 resulted in 0.34–6.25 ng/ml, and sensitivity was 0.30 ng/ml. The interassay and intra-assay precisions were <10%.

Serum anti-Hsp70 and anti-Hsp60 antibodies

Titers of anti-Hsp70 and anti-Hsp60 antibodies in serum samples (diluted 1:1,000) were measured using two commercial enzymoimmunoassays (EKS-750 and EKS-650, Assay-Designs-Stressgen, Ann Arbor, MI, USA) in a microplate coated with recombinant human Hsp70 or Hsp60, capturing anti-Hsp70 and anti-Hsp60 antibodies present in serum (the test detects all immunoglobulin isotypes, IgA, IgG, IgM). HSP antibody concentrations were expressed as microgram per milliliter. The working range (linearity) for anti-Hsp70 resulted in 31.25–1,000 μg/ml, and sensitivity was 6.79 μg/ml. The interassay and intra-assay precisions were <10%. For anti-Hsp60, the linearity was 7.81–250 μg/ml, the sensitivity was 6.79 μg/ml, and the interassay and intra-assay precisions were <10%.

Statistical analysis

To examine the distribution of data, a Kolmogorov–Smirnoff test was applied. As data were not normally distributed, results were analyzed using their medians, ranges, and interquartile ranges. Variable medians were compared using nonparametric tests: Mann–Whitney's *U* test for two independent samples and Kruskal–Wallis test for three independent samples. Qualitative and quantitative variables were analyzed using Spearman's correlation test. All probability values are derived from two-tailed analyses, and the statistical significance level selected was $p<0.05$. SPSS 16.0 software for Windows was used for statistical analysis (SPSS Inc., Chicago, IL, USA).

Results

The study population consisted of 456 subjects randomly selected and stratified by gender, who were permanent employees at HGUGM and who met the inclusion criteria and provided their informed consent. Only four subjects had to be excluded from the study for various reasons (0.088%), for which reason a total of 452 subjects were included, of which 234 were females (51.8%) with a mean age of 49.95±6.89 (x ± SD) years and 218 were males (48.2%) with a mean age of 48.86±7.27 years. After applying the Task Force's Coronary Risk Chart (Wood et al. 1998), the subjects participating in the study were distributed as shown in Table 1.

Table 2 shows the prevalence of classic vascular risk factors according to the criteria established by Wood et al. 1998.

Serum levels of Hsp70 (HSPA1A) were detectable in 444 subjects, representing 98.23% of cases. The median values of circulating Hsp70 (HSPA1A) were 1.21 ng/ml (range 0.00–29.55 mg/ml). The values were lower among women (median = 1.19 ng/ml, range 0.00–6.76 ng/ml) than among men (median = 1.26 ng/ml, range=0.26–29.55 ng/ml), which has a marginal statistical significance ($p<0.1$ Mann–Whitney test). When the population was segmented according to vascular risk, the concentration of circulating Hsp70 (HSPA1A) was significantly lower in the group with established atherosclerotic disease than in the other two groups combined ($p<0.05$, Kruskal–Wallis test) and in group 0 (Mann–Whitney test $p=0.010$) and group 1 (Mann–Whitney test $p=0.010$) separately (Table 3). Among the subjects in group 0, the concentration of Hsp70 (HSPA1A) was significantly lower in women than in men (data not shown).

Table 1 Frequency of cases per vascular risk group, by gender

Gender	Group	Frequency	Percent
Female	Group 0	149	63.7
	Group 1	65	27.8
	Group 2	20	8.5
	Total	234	100.0
Male	Group 0	90	41.3
	Group 1	96	44.0
	Group 2	32	14.7
	Total	218	100.0

Group 0 subjects with no vascular risk factor or a risk lower than 5%, *Group 1* subjects with moderate vascular risk (10–20%) who do not have established disease, *Group 2* subjects with established atherosclerotic disease

In contrast, Hsp60 (HSPB1) was detectable in only <5% of the population studied, for which reason no analysis was done.

There were detectable levels of anti-Hsp70 and anti-Hsp60 antibodies in all study subjects. The median concentration of anti-Hsp70 antibodies in the global population was 339.84 μg/ml (range 48.50–2,796.81 μg/ml), with no gender differences. When the subjects were subdivided according to their degree of vascular risk, patients with some kind of atherosclerotic disease had the lowest concentration of circulating anti-Hsp70 antibodies that was statistically significant when compared to disease-free subjects ($p=0.026$, Mann–Whitney test). Patients with moderate vascular risk, group 1, showed also a significant decrease of anti-Hsp70 with respect to group 0 ($p=0.007$, Mann–Whitney test; Table 3).

The serum concentration of anti-Hsp60 antibodies in the study population showed a median of 39.69 μg/ml (range 10.01–346.50 μg/ml), with no gender differences. This concentration was higher in the group with no vascular risk or with risk <5% (group 0) than in the rest of groups, and it was statistically significant compared with group 1 (sub-

Table 2 Prevalence of classic vascular risk factors in the general population

Vascular risk factor			Frequency	Percent
Hypercholesterolemia	Chol>200 mg/dl		256	56.6
	LDL>130 mg/dl		219	48.45
Hypertriglyceridemia			70	15.5
Hyperglycemia			38	8.4
Hypertension			103	22.8
Smoking status[a]		0	312	69.0
		1	84	18.6
		2	50	11.1
		3	6	1.3
Alcohol[b]		0	288	63.7
		1	141	31.2
		2	22	4.9
		3	1	0.2

[a] Smoking degree: 0, nonsmoker; 1, 0–20 cigarettes per day; 2, 20–40 cigarettes per day; 3,>40 cigarettes per day

[b] Alcohol intake degree: 0, none or less than 20 g of alcohol per day; 1, 20–40 g of alcohol per day; 2, 40–80 g of alcohol per day; 3, >80 g of alcohol per day

jects with moderate vascular risk who do not have established disease; $p=0.039$, Mann–Whitney test), but not with group 2 (Table 3). We presume that the value of anti-Hsp60 in group 2 can become statistically significant if we increase the sample size of this group.

A correlation has not been demonstrated between the concentrations of circulating Hsp70 (HSPA1A), anti-Hsp70, and anti-Hsp60 and classical vascular risk factors and C-reactive protein. However, there is a correlation between serum levels of anti-Hsp70 antibodies and Hsp70 (HSPA1A; $p<0.005$, Spearman's correlations) and between anti-Hsp70 and anti-Hsp60 antibodies ($p<0.001$; Fig. 1).

Discussion

Because of the random character of the population selection, the results obtained in relation to the cardiovascular health state might be applicable to the general population. All the individuals included in the study, with the exception of the 52 patients with cardiovascular disease, were, a priori, healthy people. Nevertheless, after applying the Task Force's Coronary Risk Chart (Wood et al. 1998), 35.2% of them had a moderate risk, 59.6% of which were males, without previous knowledge of it. This corroborates the idea of the lack of awareness of the cardiovascular health state of the population which contributes to the silent development of the cardiovascular disease and supports the need for the search of biomarkers of vascular risk that could

Table 3 Circulating levels of Hsp70 (HSPA1A) and autoantibodies to Hsps in the study population, grouped according to vascular risk

	Group 0		Group 1		Group 2		p
Hsp70 (HSPA1A) (ng/ml)	$N=235$	1.26 (0.19–29.55)	$N=158$	1.22(0.00–7.54)	$N=51$	1.09 (0.05–3.90)	0.028*
Anti-Hsp70 (μg/ml)	$N=239$	354.72 (63.00–2796.81)	$N=161$	311.84 (66.99–2002.87)	$N=52$	297.22 (48.50–1180.60)	0.007**
Anti-Hsp60 (μg/ml)	$N=239$	42.03 (10.01–346.50)	$N=161$	37.89 (11.15–244.20)	$N=52$	39.61 (13.70–191.90)	0.039***

Results given as median (range).

Group 0 subjects with no vascular risk factor or a vascular risk lower than 5%, *Group 1* subjects with moderate vascular risk (10–20%) who do not have established disease, *Group 2* patients with established atherosclerotic disease

*$p<0.05$ group 2 vs group 0 and group 1 combined (Kruskal–Wallis test) and separately: group 2 vs group 0 (Mann–Whitney test $p=0.010$) and group 2 vs group 1 (Mann–Whitney test $p=0.010$)

**$p<0.05$ Kruskal–Wallis test. Group 2 vs group 0, $p=0.026$ Mann–Whitney test; group 1 vs group 0 $p=0.007$ Mann–Whitney test

***$p<0.05$ group 0 vs group 1 (Mann–Whitney test)

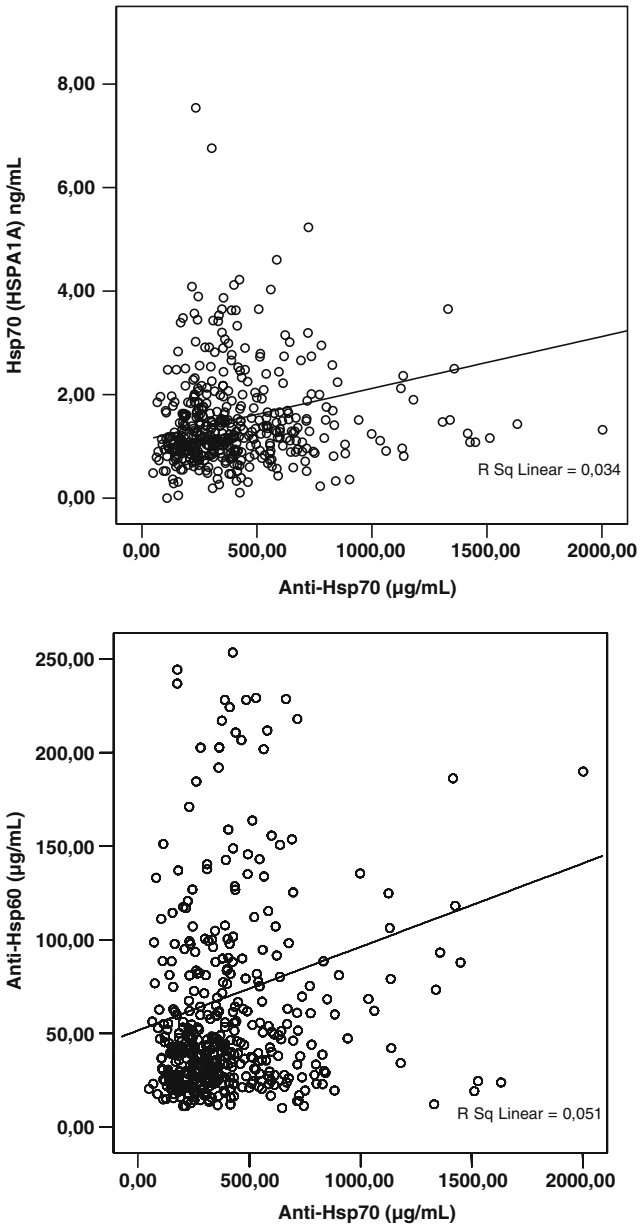

Fig. 1 Correlation of anti-Hsp70 autoantibodies with Hsp70 (HSPA1A) and anti-Hsp60 autoantibodies ($p<0.001$)

be applied in a simple and systematic way to the population.

The present study showed significantly lower levels of circulating Hsp70 (HSPA1A) in patients suffering from some type of atherosclerotic disease, regardless of conventional risk factors and inflammation markers. This agrees with prior studies (Pockley et al. 2003; Zhu et al. 2003; Marin-Ventura et al. 2007; Armutcu et al. 2008) and corroborates the hypothesis that circulating Hsp70 (HSPA1A) should be considered a biomarker for the progression of atherosclerosis. Additionally, low levels of serum Hsp70 (HSPA1A) have been shown in patients with metabolic syndrome (Armutcu et al. 2008) and with type 2 diabetes (Hooper and Hooper 2009). The lower concentration of circulating Hsp70 (HSPA1A) in women confirms epidemiological data showing worse prognosis of cardiovascular accidents for women (Silander et al. 2008). The unanswered question, asked by Hooper and Hoopet 2004, remains: are low levels of Hsp70 the cause or the consequence of atherosclerosis? Taking into consideration that Hsp70 (HSPA1A) has anti-inflammatory properties due to the inhibition of the expression of proinflammatory cytokines (Luo et al. 2008) and of proinflammatory transcription factors such as the nuclear κβ factor by blocking target, activation, and binding (Stice and Knowlton 2008), low levels of Hsp70 (HSPA1A) would promote a proinflammatory state and increase the vulnerability of the arterial wall to the damaging action of vascular risk factors involved in endothelial dysfunction, the first stage in the development of the atherosclerotic plaque. On the other hand, low levels of Hsp70 (HSPA1A) may be the result of the endothelial dysfunction that entails a reduction in the production of NO, a powerful inducer of protein synthesis (Pockley et al. 2003). Marin-Ventura et al. (2007) submit that the reduction in levels of circulating Hsp70 (HSPA1A; which they verified) in patients with atherosclerotic disease may be the result of the degradation of the protein by proteases secreted by activated neutrophils present in the atheromatous plaque and that the concentration of circulating Hsp70 (HSPA1A) reflects the balance between its secretion by healthy arteries and its degradation by atherosclerotic proteases. In contrast to these studies, Zhang et al. (2010) reported that high Hsp70 levels were associated with the increased risk and severity of acute coronary syndrome, levels that gradually decreased in the 7 days of recovery. Differences in population characteristics between our study—chronic atherosclerotic patients—and that of Zhang et al. (2010)—patients with acute coronary heart disease—might explain the discrepant findings.

In the present study, patients with a moderate vascular risk (10–20%) have a concentration of circulating anti-Hsp70 antibodies lower than the control group, and this reduction is highest and statistically significant in the group of atherosclerotic patients. Hsp70 is a protein with powerful immunological properties: it has been associated with the innate immune response (Triantafilou et al. 2008), and a strong binding to B cells, which are responsible for antibody production, has been shown (Asea 2005). Although there are several studies that link immunity to Hsp70 in the promotion of chronic inflammatory conditions such as multiple sclerosis, diabetes, and cardiovascular disease, many other studies show that such immune response attenuates inflammatory disease (Wieten et al. 2007). Specifically, it has been shown that patients with stable and unstable angina (Herz et al. 2006) and with acute

coronary syndrome (Zhang et al. 2010) have lower levels of anti-Hsp70 antibodies than the healthy population and that microvascular and macrovascular complications in patients with type 1 diabetes positively correlate with lower levels of anti-Hsp70 and anti-Hsp60 antibodies (Gruden et al. 2009). The decrease in circulating anti-Hsp70 antibodies in atherosclerotic patients may be explained by the simultaneous decrease in the levels of extracellular Hsp70 (HSPA1A)—parameters which also show a significantly positive correlation. A low serum concentration of Hsp70 (HSPA1A) may be lower than the threshold necessary to induce immune response (Pockley et al. 2009), which would determine a lesser synthesis of anti-Hsp70 autoantibodies. For Herz et al. (2006), the lower levels of anti-Hsp70 antibodies in patients with vascular disease may reflect the formation of heat shock protein 70–heat shock protein 70 antibody immune complexes within the atherosclerotic plaque, which would potentially contribute to the progression of the plaque. Another explanation is that serum Hsp70 (HSPA1A) and anti-Hsp70 antibodies could be forming antigen–antibody complexes, thus decreasing detectable levels of circulating anti-Hsp70 antibodies (Pockley and Multhoff 2008b; Zhang et al. 2010).

In our study, serum levels of Hsp60 (HSPD1) were not detectable in most cases, for which reason it is not possible to discuss its potential role in the resulting atherosclerosis. Similar to Gruden et al. (2009), the percentage of subjects with detectable levels of Hsp60 (HSPD1) was under 10%; we agree with these authors that this is not due to methodological reasons because our serum samples were adequately stored and the same samples were satisfactorily used to quantify Hsp70 (HSPA1A). The population without vascular risk factors or with a risk <5% had higher concentrations of anti-Hsp60 antibodies. The precise role of Hsp60 (HSPD1) in the pathogenesis of atherosclerosis is not well defined; however, in contrast to Hsp70 (HSPA1A), it seems to have proinflammatory properties (it has a direct correlation with levels of TNF-α, Lewthwaite et al. 2002a, b, and stimulates the release of IL-6 and IL-8 in adipocytes, Gülden et al. 2008) and has atherogenic properties: increased levels of circulating Hsp60 (HSPD1) has been shown in subjects with borderline hypertension and intima thickening in the carotid artery (Pockley et al. 2000) and in atherosclerotic subjects (Xu et al. 2000, Xiao et al. 2005). More recently, Zhang et al. (2008b) showed, in a wide case–control study, a strong positive correlation between high levels of Hsp60 (HSPD1) and the risk of CHD and that this connection does not depend on conventional CHD risk factors. In the same study, in a prospective observational study about the influence of acute myocardial infarction (AMI) on circulating Hsp60 (HSPD1) levels, they detected elevated Hsp60 levels in patients on the day of arrival and the following day, probably due to either the necrosis of cardiomyocytes and concomitant endothelial dysfunction or AMI-related shear stress.

Hsp60 (HSPD1) is targeted by T and B cells in spontaneous autoimmune NOD diabetes and in autoimmune diabetes patients (Szebeni et al. 2005). Elevated levels of anti-Hsp60 antibodies in several vascular diseases (Mehta et al. 2005) and specifically in patients with CHD (Zhang et al. 2008a) have been described. Our finding of lower levels of anti-Hsp60 antibodies in initial stages of atherosclerosis (vascular risk 10–20%) may be due to the expression of Hsp60 (HSPD1) on the surface of endothelial cells stressed by the damaging action of various vascular risk factors; Hsp60 (HSPD1) may act as a target epitope for the attack of anti-Hsp60 antibodies stimulated by infection or other stress situations (Wick et al. 2004), generating immune complexes on the arterial wall and reducing the levels of detectable circulating antibodies. The concentration of anti-Hsp60 antibodies would be an independent marker of the degree of arterial inflammation, rather than of atherosclerotic lesion. In order to compare our results about the anti-Hsp60 concentrations in patients with established atherosclerosis with the data of Zhang et al. (2008a, b) who described, in a very large case–control study, the association of high concentrations of anti-Hsp60 antibodies and CHD risk, we need to increase the sample size of the patient group to come to some definitive conclusions. To calculate the sample size, it is important to bear in mind that a lot of patients take multiple medications like statins and that statins, on the one hand, improve vascular endothelial cell function, inducing heat shock factor 1 and heat shock proteins synthesis (Uchiyama et al. 2006), and on the other hand, subjects under statin treatment have lower antibody concentration to Hsp70, Hsp65, and Hsp70 than subjects without treatment, probably because of their immunomodulatory properties (Ghayour Mobarban et al. 2005; Guisasola et al. 2009).

In conclusion, serum Hsp70 (HSPA1A) levels themselves are inversely related to the development of atherosclerosis. More prospective studies, like that of Pockley et al. (2003), are necessary to define whether the level of Hsp70 (HSPA1A) is a biomarker of the progression of the disease or has an active role in the endothelial lesion, in which case it would acquire the features of a “candidate” risk factor, according to the definition of Pearson et al. (1996). In view of the apparent atheroprotective effect of extracellular Hsp70, potential therapeutic tools to increase its level should be researched and should include not only new therapeutic targets but also measures as simple as changes in lifestyle, including physical exercise (more intense in women), which have proven effects, increasing the levels of circulating Hsp70 (Milne and Noble 2008). The reduction in the concentration of anti-Hsp70 antibodies constitute a second independent biomarker of vascular disease, and levels of

circulating anti-Hsp60 antibodies may constitute, per se, a marker of inflammation in atherosclerosis.

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References

Adult Treatment Panel III (2001) Executive summary of the third report of the National Cholesterol Education Program (NCEP). Expert Panel on Detection, Evaluation and treatment of high blood cholesterol in adults. JAMA 285:2486–2497

Armutcu F, Ataymen M, Atmaca H, Gurel A (2008) Oxidative stress markers, C-reactive protein and heat shock protein 70 levels in subjects with metabolic syndrome. Clin Chem Lab Med 46:785–790

Asea A (2005) Stress proteins and initiation of immune response: chaperokine activity of hsp72. Exserc Immunol Rev 11:34–45

Asea A (2008) Hsp70: a chaperokine. In: Chadwick DJ, Goode J (eds) The biology of extracellular molecular chaperones. Wiley, Chichester, pp 173–179, discussion 179–183, 221–224; Novartis Found Symp, West Sussex, UK

Binder CJ, Hartvigsen K, Chang MK, Miller M, Brode D, Palinski W et al (2004) IL 5 links adaptative and natural immunity specific for epitopes of oxidized LDL and protects from atherosclerosis. J Clin Invest 114:427–437

Calderwood SK (2005) Chaperones and slow death—a recipe for tumor immunotherapy. Trends Biotechnol 23:57–59

D'Agostino RB Sr, Gruñid SM, Sullivan LM, Wilson P (2001) Validation of the Framingham coronary heart disease prediction scores: results of a multiple ethnic groups investigation. JAMA 286:180–187

Dawber TR, Meadors GF, Moore FEJ (1951) Epidemiological approaches to heart disease: the Framingham study. Am J Public Health 41:279–286

George J, Harats D, Gilburd B, Afek A, Shaish A, Kopolovic J et al (2000) Adoptive transfer of beta (2) glycoprotein I reactive lymphocytes enhances early atherosclerosis in LDL receptor deficient mice. Circulation 102:1822–1827

Ghayour Mobarban M, Lamb DJ, Lovell DP, Livingstone C, Wang T, Ferns GAA (2005) Plasma antibody titres to heat shock proteins 60, 65 and 70: their relationship to coronary risk factors in dyslipidaemic patients and healthy individuals. Scand J Clin Lab Invest 65:601–614

Graham I et al (2007) European guidelines on cardiovascular disease prevention in clinical practice: executive summary. Fourth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (Constituted by representatives of nine societies and by invited experts). Eur J Cardiovasc Prev Rehabil 14(Suppl 2):E1–E40

Grau M, Marrugat J (2008) Risk functions and the primary prevention of cardiovascular disease. Rev Esp Cardiol 61:404–416

Gruden G, Bruno G, Chaturvedi N, Burt D, Pinach S, Schalkwijk C, Stehouwer CD, Witte DR, Fuller JH, Cavallo-Perin P, EURODIAB Prospective Complications Study Group (2009) ANTI-HSP60 and ANTI-HSP70 antibody levels and micro/macrovascular complications in type 1 diabetes: the EURODIAB Study. J Intern Med 266:527–536

Grundy SM, Pasternak R, Greenland P, Smith S Jr, Fuster V (1999) Assessment of cardiovascular risk by use of multiple-risk-factor assessment equations: a statement for healthcare professionals from the American Heart Association and the American College of Cardiology. Circulation 100:1481–1492

Guisasola MC, Dulin E, Almendral J, García-Barreno P (2009) Reduction of heat shock protein antibody levels by statin therapy. Lipids 44:317–324

Gülden E, Mollérus S, Brüggemann J, Burkart V, Habich C (2008) Heat shock protein 60 induces inflammatory mediators in mouse adipocytes. FEBS Lett 582:2731–2736

Han Z, Truong QA, Park S, Breslow JL (2003) Two Hsp70 family members expressed in atherosclerotic lesions. Proc Natl Acad Sci U S A 100:1256–1261

Herz I, Rosso R, Roth A, Keren G, George J (2006) Serum levels of anti heat shock protein 70 antibodies in patients with stable and unstable angina pectoris. Acute Card Care 8:46–50

Hooper PL, Hooper PL (2009) Inflammation, heat shock proteins and type 2 diabetes. Cell Stress Chaperones 14:113–115

Hooper PL, Hoopet JJ (2004) Is low-heat shock protein 70 a primary or a second event in the development of atherosclerosis? Hypertension 43:e18

Jara LJ, Medina G, Vera Lastra O, Amigo MC (2006) Accelerated atherosclerosis, immune response and autoimmune rheumatic diseases. Autoimmun Rev 5:195–201

Jin X, Wang R, Xiao C et al (2004) Serum and lymphocyte levels of heat shock protein 70 in aging: a study in the normal Chinese population. Cell Stress Chaperones 9:69–75

Kampinga HH, Hageman J, Vos MJ et al (2009) Guideline for the nomenclature of the human heat shock proteins. Cell Stress Chaperones 14:105–111

Kanwar RK, Kanwar JR, Wang D, Ormrod DJ, Krissansen GW (2001) Temporal expression of heat shock proteins 60 and 70 at lesion-prone sites during atherogenesis in ApoE-deficient mice. Arterioscler Thromb Vasc Biol 21:1991–1997

Kaperonis EA, Liapis CD, Kakisis JD, Dimitroulis D, Papavassiliou VG (2006) Inflammation and atherosclerosis. Eur J Vasc Endovasc Surg 31:386–393

Lahoz C, Mostaza JM (2007) La ateroesclerosis como enfermedad sistémica. Rev Esp Cardiol 60:184–195

Lewthwaite J, Owen N, Coates A, Henderson B, Steptoe A (2002a) Circulating human heat shock protein 60 in the plasma of British civil servants: relationship to physiological and psychosocial stress. Circulation 106:196–201

Lewthwaite J, Owen N, Coates A, Henderson B, Steptoe A (2002b) Circulating human heat shock protein 60 in the plasma of British civil servants: relationship to physiological and psychosocial stress. Circulation 106:196–201

Luo X, Zuo X, Zhou Y, Zhang B, Shi Y, Liu M et al (2008) Extracellular heat shock protein 70 inhibits tumour necrosis factor-alpha induced proinflammatory mediator production in fibroblast-like synoviocytes. Arthritis Res Ther 10:R41. doi:10.1186/ar2399

Marin-Ventura JL, Leclerq A, Blanco-Colio LM et al (2007) Low plasma levels of HSP70 in patients with carotid atherosclerosis are associated with increased levels of proteolytic markers of neutrophil activation. Atherosclerosis 194:334–341

Mehta TA, Greenman J, Ettelaie C, Venkatasubramaniam A, Chetter IC, McCollum PT (2005) Heat shock proteins in vascular disease. A review. Eur J Vasc Endovasc Surg 29:395–402

Milne KJ, Noble EG (2008) Response of the myocardium to exercise: sex-specific regulation of Hsp70. Med Sci Sports Exerc 40:655–663

Molvarec A, Tamási L, Losonczy G, Madách C, Prohászka Z, Rigó J (2009) Circulating heat shock protein 70 (HSPA1A) in normal and pathological pregnancies. Cell Stress Chaperones 15:237–247. doi:10.1007/s12192-009-0146-5

Pearson TA, McBride PE, Miller NH, Smith SC (1996) 27th Bethesda Conference: matching the intensity of risk factor management

with the hazard for coronary disease events. Task Force 8. Organization of Preventive Cardiology Service. J Am Coll Cardiol 27:1039–1047

Pockey AG, Georgiades A, Thulin T, Faide U, Frostegård J (2003) Serum heat shock protein 70 levels predict the development of atherosclerosis in subjects with established hypertension. Hypertension 42:235–238

Pockley AG, Multhoff G (2008) Cell stress proteins in extracellular fluids: friend or foe? In: Chadwick DJ, Goode J (eds) The biology of extracellular molecular chaperones. Wiley, Chichester, pp 86–95, discussion 96–100; Novartis Found Symp, West Sussex, UK

Pockley AG, Shepherd J, Corton JM (1998) Detection of heat shock protein 70 (Hsp70) and anti-Hsp70 antibodies in the serum of normal individuals. Immunol Invest 27:367–377

Pockley AG, Bulmer J, Hanks BM, Wright BH (1999) Identification of human heat shock protein 60 (Hsp60) and anti-Hsp60 antibodies in the peripheral circulation of normal individuals. Cell Stress Chaperones 4:29–35

Pockley AG, Wu R, Lemme C et al (2000) Circulating heat shock protein 60 is associated with early cardiovascular disease. Hypertension 36:303–307

Pockley AF, Georgiades A, Thulin T, Faire U, Frostegård J (2003) Response: is low-heat shock protein 70 a primary or a secondary event in the development of atherosclerosis? Hypertension 43:e18–e19

Pockley AG, Muthana M, Calserwood SK (2008) The dual immunoregulatory roles of stress proteins. Trends Biochem Sci 33:71–79

Pockley AG, Calderwood SK, Multhoff G (2009) The atheroprotective properties of Hsp70: a role for Hsp70–endothelial interactions? Cell Stress Chaperones 14:545–553

Ross R (1999) Atherosclerosis—an inflammatory disease. N Engl J Med 340:115–126

Ross R, Glomset JA (1976) The pathogenesis of atherosclerosis. N Engl J Med 295:369–377, see also pp 420–425

Shamaei-Tousi A, Halcox JP, Henderson B (2007) Stressing the obvious? Cell stress and cell stress proteins in cardiovascular disease. Cardiovasc Res 74:19–28

Silander K, Alanne M, Kristiansson K, Saarela O, Ripatti S, Auro K et al (2008) Gender differences in genetic risk profiles for cardiovascular disease. PLoS ONE 3:e3615

Stice JP, Knowlton AA (2008) Estrogen, NFkappaB, and the heat shock response. Mol Med 14:517–527

Szebeni A, Schloot N, Kecskeméti V, Hosszúfalusi N, Pánczél P, Prohászka Z et al (2005) Th1 and Th2 cell responses of type 1 diabetes patients and healthy controls to human heat-shock protein 60 peptides AA437-460 and AA394-408. Inflamm Res 54:415–419

The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (1997) Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Diabetes Care 20:1183–1197

Triantafilou M, Sawyer D, Nor A, Vakakis W, Triantafilou K (2008) Cell surface molecular chaperones as endogenous modulators of the innate immune response. In: Chadwick DJ, Goode J (eds) The biology of extracellular molecular chaperones. Wiley, Chichester, pp 74–79, discussion 79–85; Novartis Found Symp, West Sussex, UK

Uchiyama T, Atsuta H, Utsugi T et al (2006) Simvastatin induces heat shock factor 1 in vascular endothelial cells. Atherosclerosis 188:265–273

Wick G, Knoflach M, Xu Q (2004) Autoimmune and inflammatory mechanisms in atherosclerosis. Annu Rev Immunol 22:361–403

Wieten L, Broere F, van der Zee R, Koerkamp EK, Wagenaar J, van Eden W (2007) Cell stress induced HSP are targets of regulatory T cells: a role for HSP inducing compounds as anti-inflammatory immuno-modulators? FEBS Lett 581:3716–3722

Wood D, De Backer G, Faergeman O, Graham I, Mancia G, Pyorala K (1998) Prevention of coronary heart disease in clinical practice: recommendations of the Second Joint Task Force of European and others societies on coronary Prevention. Atherosclerosis 140:199–270

Xiao Q, Mandal K, Schett G et al (2005) Association of serum-soluble heat shock protein 60 with carotid atherosclerosis: clinical significance determined in a follow-up study. Stroke 36:2571–2576

Xu Q (2002) Role of heat shock proteins in atherosclerosis. Arterioscler Thromb Vasc Biol 22:1547–1559

Xu Q, Schett G, Perschinka H et al (2000) Serum soluble heat shock protein 60 is elevated in subjects with atherosclerosis in a general population. Circulation 102:14–20

Zhang X, He MA, Cheng L et al (2008a) Joint effects of antibody to heat shock protein 60, hypertension, and diabetes on risk of coronary heart disease in Chinese. Clin Chem 54:1046–1052

Zhang X, He M, Cheng L et al (2008b) Elevated heat shock protein 60 levels are associated with higher risk of coronary heart disease in Chinese. Circulation 118:2687–2693

Zhang X, Xu Z, Zhou L et al (2010) Plasma levels of Hsp70 and anti-Hsp70 antibody predict risk of acute coronary syndrome. Cell Stress Chaperones. doi:10.1007/s12192-010-0180-3

Zhu J, Quyyumi AA, Wu H et al (2003) Increased serum levels of heat shock protein 70 are associated with low risk of coronary artery disease. Arterioscler Thromb Vasc Biol 23:1055–1059