Circulating Human Heat Shock Protein 60 in the Plasma of British Civil Servants

Relationship to Physiological and Psychosocial Stress

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Background—The Whitehall cohort studies (I and II) of British civil servants have identified sociodemographic, psychosocial, and biological risk factors for coronary heart disease (CHD). To identify mechanisms responsible for susceptibility to CHD, specific biological markers of stress are increasingly being measured. One marker linked to susceptibility to CHD is heat shock protein (Hsp) 60.

Methods and Results—Blood was taken from 229 civil servants (126 men and 103 women) in the Whitehall II cohort drawn equally from the range of employment grades. Plasma was assayed for levels of Hsp60, tumor necrosis factor α (TNFα), C-reactive protein, von Willebrand factor, high density lipoprotein (HDL), total cholesterol, and total/HDL ratio. Psychosocial measures included socioeconomic status, psychological distress, and social isolation. The majority of the participants had Hsp60 in their plasma, and ~20% had >1000 ng/mL of this protein (a concentration likely to induce biological effects). A positive association between plasma Hsp60 and TNFα and a negative association with von Willebrand factor was found. There was also a significant association between elevated Hsp60 levels, low socioeconomic status, and social isolation, together with an association with psychological distress in women.

Conclusions—The majority of participants exhibited Hsp60 in their plasma, and there was evidence of an association between levels of this stress protein and the proinflammatory cytokine, TNFα, and with various psychosocial measures.

Key Words: coronary disease ■ cytokines ■ lifestyle ■ proteins ■ stress

The longitudinal Whitehall cohort studies (I and II) of British civil servants have helped to identify risk factors for coronary heart disease (CHD).1,2 Increasingly, specific biological markers of stress are being measured in an attempt to identify mechanisms responsible for susceptibility to CHD. One marker linked to CHD susceptibility is the inducible heat shock protein (Hsp) 60, or chaperonin (Cpn) 60.3 This normally intracellular protein is a very potent immunogen, and infection with bacteria is associated with significant lymphocyte reactivity to bacterial Cpn60.4 Immunological cross-reactivity between bacterial Cpn60 and the homologous human Cpn60 (termed Hsp60), which is present on the surface of mechanically stressed vascular endothelial cells, has been proposed to be involved in the origin of atherosclerosis.3,5

The Hsp60 protein is an oligomeric molecule found in mitochondria.6 Thus, reports that this protein is found in other cellular locations, including the plasma membrane, are surprising.6 In recent years, Hsp60 has been found in the serum of healthy individuals7 and those with cardiovascular disease.8,9

In this study, we have examined healthy middle-aged individuals taking part in the Whitehall II study to determine the presence of Hsp60 in their plasma and its relationship to markers of inflammation and cardiovascular risk and to psychosocial factors linked with cardiovascular disease risk.

Methods

Participants

A total of 229 civil servants (126 men and 103 women) in the Whitehall II cohort drawn equally from high, intermediate, and low employment grades were recruited using the following criteria: white, aged 45 to 58 years, no history of CHD or previous diagnosis or treatment for hypertension, and willingness to participate in ambulatory blood pressure monitoring. The study was approved by an institutional review committee, and subjects gave informed consent. All procedures followed were in accordance with institutional guidelines.

Procedures

Participants attended the laboratory either at 9:30 AM or 2:00 PM and were instructed not to have drunk caffeinated beverages or to have smoked for at least 2 hours before the study and not to have...
consumed alcohol or have exercised on the evening before or the day of testing. Participants were not suffering from any bacterial or protozoal infection, and sessions were rescheduled if illness was reported. At the beginning of the session, height and weight were measured. A venous cannula was inserted, and the participant rested for 30 minutes before blood samples were drawn. Blood was collected in EDTA tubes and centrifuged at 1500g for 10 minutes, and plasma was collected and stored at –80°C. Blood pressure was measured under resting conditions.

Psychosocial Measures
Socioeconomic status was indexed by income, and participants were divided into those with incomes above or below £35 000 per annum. Psychological distress was assessed using the General Health Questionnaire (GHQ) 30-item version.10 The recommended clinical threshold of 4/5 was used to define psychological distress,11 with a positive score indicating significant psychological distress. Social isolation was measured with questions concerning contact with family, friends, and relatives that were derived from the Close Persons Questionnaire.12 For the purposes of the analysis, the sample was divided into respondents with low (54.2%), medium (35.7%), and high (10.1%) social isolation scores.

Assay of Hsp60 in Plasma
Hsp60 was measured by 2-site ELISA. Briefly, 96-well plates (Nunc Maxisorp) were coated with mouse monoclonal anti-Hsp60 antibody (clone LK-1, a kind gift of Dr. Peter van Kooten, University of Utrecht, Utrecht, the Netherlands) at 0.5 µg/mL in PBS overnight at 4°C. Plates were washed in wash buffer (0.5 mol/L NaCl, 2.5 mmol/L Na2HPO4, 7.5 mmol/L NaH2PO4, and 0.1% Tween 20), and nonspecific binding sites were blocked by incubation with 1% bovine serum albumin (Sigma) in wash buffer for 1 hour at room temperature. After washing, recombinant Hsp60 (Stressgen; 0 to 2500 ng/mL) or dilutions of human plasma were added, and plates were incubated overnight at 4°C and then washed. Bound Hsp60 was detected by incubation with biotinylated goat polyclonal antibody to Hsp60 (clone N-20, Santa Cruz Biotechnology) at 1/1000 for 1 hour at 37°C. Plates were washed and incubated with poly-HRP-conjugated streptavidin (CLB) at 1/10 000 for 30 minutes at 37°C. Binding of conjugated antibody was detected using 1.2-phenylene-diamine dihydrochloride (OPD) substrate. The reaction was stopped with 1 mol/L H2SO4, and absorbance was determined at 492 nm using a Dynex plate reader. Each plasma sample was assayed in triplicate and at least 2 separate assays. Plasma from the patient with the highest level of soluble Hsp60, was added to the column. Unbound plasma constituents were washed from the column using PBS containing 0.1% Tween 20. Bound proteins were eluted from the column using 0.1 mol/L glycine-HCl (pH 2.5). Samples were electrophoresed, and protein bands were stained with colloidal Coomassie blue (Sigma). A replicale gel was transferred to nitrocellulose and incubated with an anti-Hsp60 antibody followed by HRP-conjugated secondary antibodies, and Hsp60 was detected using enhanced chemiluminescence (Amersham Pharmacia Biotech).

Immunooaffinity Chromatography and Detection of Proteins Bound to Anti-Hsp60 Antibody
Mouse monoclonal antibody LK-1 was coupled to Affi-Gel 10 (Bio-Rad) according to the manufacturer’s instructions. After washing in coupling buffer, filtered plasma from individual number 228, with the highest recorded level of soluble Hsp60, was added to the column. Unbound plasma constituents were washed from the column using PBS containing 0.1% Tween 20. Bound proteins were eluted from the column using 0.1 mol/L glycine-HCl (pH 2.5). Samples were electrophoresed, and protein bands were stained with colloidal Coomassie blue (Sigma). A replicale gel was transferred to nitrocellulose and incubated with an anti-Hsp60 antibody followed by HRP-conjugated secondary antibodies, and Hsp60 was detected using enhanced chemiluminescence (Amersham Pharmacia Biotech).

Assay of Additional Plasma Constituents
Total plasma cholesterol and high-density lipoprotein (HDL) cholesterol were assessed using standard methods. Tumor necrosis factor α (TNFα) was measured using a 2-site ELISA (R and D Systems). C-reactive protein and von Willebrand factor were assayed by 2-site ELISAs (Duko Diagnostics).

Statistical Analysis
Because the distribution of Hsp60 concentration was markedly skewed, associations with psychosocial and other biological measures were analyzed in 2 ways. First, Hsp60 values were log-transformed before ANOVA, with psychosocial and other biological measures as independent variables. C-reactive protein, TNFα, HDL cholesterol, total cholesterol, total/HDL cholesterol ratio, von Willebrand factor, and systolic and diastolic blood pressure were each divided into tertiles to ensure a common metric. Income and psychosocial distress were analyzed as binary variables, and social isolation was analyzed with three levels. The Hsp60 results are presented as geometric means. Second, a division was made between individuals with very high (>4000 ng/mL) Hsp60 concentrations and lower levels. Associations between biological and psychosocial variables and elevated human Hsp60 were analyzed using logistic regression, and odds ratios adjusted for age, sex, and body mass index are presented. The level of statistical significance was set at P<0.05.

Results
Population Characteristics
The characteristics of the participants in this study are summarized in Table 1. Men were slightly older than women on average and had higher blood pressures, whereas women had higher HDL cholesterol levels and lower total/HDL cholesterol ratios. Levels of Hsp60 did not differ according to the time of day of testing, smoking status, hormone replacement therapy (women), or age.

Evaluation of the Hsp60 Assay
Figure 1 is a representative standard curve for the Hsp60 assay whose sensitivity was 7 ng/mL. Interassay variability was no more than 10%, and intrasample variation was generally <10%. The assay did not detect the Cpn60 proteins from Escherichia coli, Mycobacterium tuberculosis, or M bovis and did not cross-react with bovine serum albumin, human fibrinogen, or E coli lipopolysaccharide. There was full recovery of exogenously added Hsp60 from 2 different
plasma samples. Western blotting of human plasma with the highest Hsp60 level revealed that the antibody reacted only with a 60-kDa protein. Immunoaffinity chromatography on an affinity column containing anti-Hsp60 monoclonal antibody LK-1 isolated a 60-kDa protein, which reacted with the antibody N-20 used in the ELISA (Figure 2).

**Distribution of Hsp60 Levels in Plasma**

Individual plasma levels of Hsp60 in the 229 participants is shown in Figure 3A, and the distribution of these values is shown in Figure 3B. Hsp60 was below the limit of assay detection in the plasma of 50 (21.8%) of the individuals tested, whereas 43 (18.8%) had values >4000 ng/mL. The mean Hsp60 concentration was 5798 ng/mL, and the median was 110 ng/mL, with an interquartile range of 13 to 1589 ng/mL.

**Associations Between Human Hsp60 in Plasma and Other Biological Variables**

There was no significant association between plasma Hsp60 and C-reactive protein concentration or blood pressure. However, Hsp60 was positively associated with plasma TNFα concentration. The mean Hsp60 was greater in participants with higher TNFα, and the proportion of individuals with elevated Hsp60 was also increased (Table 2). The odds of elevated Hsp60 (>4000ng/mL) in the highest tertile of TNFα were 3.46 (95% confidence interval [CI] 1.39 to 8.62) compared with the lowest tertile when adjusted for age, sex, and body mass index. The levels of Hsp60 in plasma were not associated with total cholesterol concentrations, but an inverse relationship with HDL cholesterol was observed. Tertiles of HDL cholesterol were constructed on a sex-specific basis because men and women differed in average concentration. The mean concentration of Hsp60 and the proportion of individuals with elevated Hsp60 were greater in the lowest rather than highest HDL cholesterol tertiles (Table 2). The highest HDL cholesterol tertile had substantially diminished odds of elevated Hsp60 (0.37; 95% CI, 0.14 to 0.94) in comparison with the lowest tertile. The reverse pattern was observed for total/HDL cholesterol ratio, with greater Hsp60 among individuals in the highest tertile. An inverse association between von Willebrand factor and Hsp60

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**Figure 1.** Representative standard dose-response curve for the Hsp60 ELISA. Data points are mean±SE for triplicate standards. The sensitivity of the assay, calculated as the concentration that gave an absorbance twice that of the zero standard, was 7.3 ng/mL.

**Figure 2.** Chromatography and Western blot analysis. Monoclonal antibody LK-1 was coupled to Affi-Gel 10 matrix by following the manufacturer’s instructions. The matrix was used to isolate Hsp60 from a plasma sample containing high Hsp60 levels, as determined by ELISA. Standard Hsp60 was loaded on the gel as a control (Hsp60). A, Isolated proteins were electrophoresed and stained with colloidal Coomassie blue. The 50-kDa band is Immunoglobulin leaking from column. B, Proteins on a replicate gel were transferred to nitrocellulose, probed with antibody, and visualized using enhanced chemiluminescence.

**Figure 3.** A, Scatter plot showing individual measurements of human Cpn60 in the plasma of the 229 civil servants. Data are expressed as mean±SEM. B, Distribution of Hsp60 in plasma of individuals from the Whitehall study (n=229). Each bar shows the number of individuals with plasma Hsp60 levels that fall into each range.
was noted. Only 9.2% of participants in the highest tertile of von Willebrand factor had elevated Hsp60, compared with 26.3% of those in the lowest tertile (adjusted odds, 0.30; 95% CI, 0.11 to 0.80).

### Association Between Hsp60 and Psychosocial Factors

Socioeconomic status, as indexed by income, was inversely associated with plasma Hsp60. The mean Hsp60 level was significantly lower in the high-income group ($P=0.033$). In addition, the odds of elevated Hsp60 in the low-income group were 3.41 (95% CI, 1.47 to 7.89) compared with the high-income group when adjusted for age, sex, and body mass index. An association between social isolation and Hsp60 was also identified. Although the mean Hsp60 in low, medium, and high isolation groups did not differ significantly, the incidence of elevated Hsp60 ($>4000$ ng/mL) was greater in the high-isolation group (Table 2). This effect remained significant after adjustment for age, sex, and body mass index.

Psychological distress, as indexed by the GHQ, was related to levels of Hsp60, but only in women. As shown in Table 2, women with scores on the GHQ above criterion had substantially elevated average Hsp60 concentrations. In addition, 37.5% of women experiencing psychological distress had elevated Hsp60, compared with 12.7% of the remainder.

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**TABLE 2. Biological and Psychosocial Factors Associated With Hsp60 Levels in Human Plasma**

<table>
<thead>
<tr>
<th></th>
<th>Hsp60, $\mu$g/mL</th>
<th>Proportion With Elevated Hsp60 ($&gt;4000$ ng/mL), %</th>
<th>Odds (95% CI) of Elevated Hsp60 ($&gt;4000$ ng/mL), Adjusted for Age, Sex, and Body Mass Index</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TNFα</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>62.4 (2.7)</td>
<td>10.8</td>
<td>1</td>
</tr>
<tr>
<td>Medium</td>
<td>81.9 (3.5)</td>
<td>17.1</td>
<td>1.82 (0.69 to 4.80)</td>
</tr>
<tr>
<td>High</td>
<td>347.2 (3.3)</td>
<td>29.2</td>
<td>3.46 (1.39 to 8.62)</td>
</tr>
<tr>
<td>$P$</td>
<td>0.006</td>
<td>0.006</td>
<td>0.008</td>
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<tr>
<td><strong>HDL cholesterol</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>226.6 (3.1)</td>
<td>24.1</td>
<td>1</td>
</tr>
<tr>
<td>Medium</td>
<td>108.0 (3.7)</td>
<td>19.7</td>
<td>0.68 (0.31 to 1.52)</td>
</tr>
<tr>
<td>High</td>
<td>58.7 (3.7)</td>
<td>13.9</td>
<td>0.37 (0.14 to 0.94)</td>
</tr>
<tr>
<td>$P$</td>
<td>0.045</td>
<td>0.079</td>
<td>0.037</td>
</tr>
<tr>
<td><strong>Total/HDL cholesterol</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>73.8 (3.5)</td>
<td>16.0</td>
<td>1</td>
</tr>
<tr>
<td>Medium</td>
<td>73.9 (3.3)</td>
<td>14.9</td>
<td>1.06 (0.42 to 2.67)</td>
</tr>
<tr>
<td>High</td>
<td>259.6 (3.4)</td>
<td>25.6</td>
<td>2.30 (0.92 to 5.74)</td>
</tr>
<tr>
<td>$P$</td>
<td>0.030</td>
<td>0.13</td>
<td>0.074</td>
</tr>
<tr>
<td><strong>Von Willebrand factor</strong></td>
<td></td>
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<tr>
<td>Low</td>
<td>188.6 (4.2)</td>
<td>26.3</td>
<td>1</td>
</tr>
<tr>
<td>Medium</td>
<td>119.0 (4.0)</td>
<td>20.5</td>
<td>0.76 (0.36 to 1.64)</td>
</tr>
<tr>
<td>High</td>
<td>70.7 (2.1)</td>
<td>9.2</td>
<td>0.30 (0.11 to 0.80)</td>
</tr>
<tr>
<td>$P$</td>
<td>0.029</td>
<td>0.011</td>
<td>0.016</td>
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<tr>
<td><strong>Income</strong></td>
<td></td>
<td></td>
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<tr>
<td>High</td>
<td>61.1 (2.8)</td>
<td>9.4</td>
<td>1</td>
</tr>
<tr>
<td>Low</td>
<td>164.4 (2.6)</td>
<td>24.1</td>
<td>3.41 (1.47 to 7.89)</td>
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<tr>
<td>$P$</td>
<td>0.033</td>
<td>0.008</td>
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<tr>
<td><strong>Social isolation</strong></td>
<td></td>
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<tr>
<td>Low</td>
<td>107.9 (2.9)</td>
<td>18.7</td>
<td>1</td>
</tr>
<tr>
<td>Medium</td>
<td>84.7 (2.64)</td>
<td>13.6</td>
<td>0.65 (0.29 to 1.44)</td>
</tr>
<tr>
<td>High</td>
<td>505.2 (6.49)</td>
<td>39.1</td>
<td>2.67 (1.06 to 7.04)</td>
</tr>
<tr>
<td>$P$</td>
<td>0.078</td>
<td>0.035</td>
<td>0.046</td>
</tr>
<tr>
<td><strong>Psychological distress</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>70.0 (2.4)</td>
<td>12.7</td>
<td>1</td>
</tr>
<tr>
<td>Positive</td>
<td>344.1 (15.0)</td>
<td>37.5</td>
<td>3.48 (1.15 to 10.5)</td>
</tr>
<tr>
<td>$P$ (women only)</td>
<td>0.047</td>
<td>0.013</td>
<td>0.027</td>
</tr>
</tbody>
</table>

Values are mean (SEM), percentage, or odds (95% CI), as indicated.
Multivariate Analysis of Factors Associated With Elevated Hsp60

The associations between plasma levels of Hsp60 and biological and psychosocial factors were identified in separate analyses, and effects may not be independent of one another. A multivariate logistic regression was therefore carried out on elevated Hsp60, including HDL cholesterol, TNFα, von Willebrand factor, income, and social isolation as predictor variables along with age, sex, and body mass index. All of the variables identified in previous sections remained independently associated with elevated Hsp60, except for social isolation. The odds (adjusted for all other factors) of elevated Hsp60 in the highest versus lowest tertile of TNFα were 4.39 (95% CI, 1.63 to 11.8; P=0.003), the odds for the highest versus lower tertile of HDL cholesterol were 0.30 (95% CI, 0.11 to 0.83; P=0.021), the odds for the highest tertile of von Willebrand factor were 0.23 (95% CI, 0.08 to 0.68; P=0.008), and the odds for the low- versus high-income group were 3.85 (95% CI, 1.53 to 9.72; P=0.004). Additional analyses, including the GHQ, was performed with women only, and the odds of elevated Hsp60 for those with positive psychological distress scores were 5.34 (95% CI, 1.11 to 25.6; P=0.036) after adjusting for all other factors. Thus, the associations between levels of plasma Hsp60 and the various biological and psychosocial risk factors seem to be mutually independent.

Discussion

Cpn60 is a highly-conserved intracellular protein essential for protein folding and a potent immunogen involved in autoimmunity. Chaperonins, both bacterial and human, also have intercellular signaling functions, but this is of biological relevance only if the chaperonin is secreted and enters the extracellular fluid. It is not known how Cpn60 proteins are released from cells, but it was recently reported that Hsp60 is present in serum. Pockley et al. assayed Hsp60 in 60 normal sera and found the range in male serum was 95 to 7573 ng/mL and in female serum, 138 to 14,679 ng/mL. Xu and coworkers assayed Hsp60 in sera from a large population of individuals in a prospective study of atherosclerosis. Approximately one-third of sera had no measurable Hsp60. In the remainder, the levels ranged as high as 11,000 ng/mL. Serum Hsp60 levels were not correlated with a range of measures including age, body mass, blood pressure, fibrinogen, and smoking. The only significant correlations were between Hsp60 and antibody titers against bacteria and circulating ceruloplasmin.

In the present study, 229 healthy individuals taking part in the Whitehall II study, a prospective study of CHD involving 10,308 London-based civil servants who were recruited in 1985 to 1988 when aged 35 to 55 years, were examined. A major difference between this and studies reported previously is that Hsp60 was detected in plasma, not serum. Preparation of serum causes biochemical changes in blood with consequent induction of cell-cell interactions and signaling events that are likely to activate cells, induce Hsp60, and stimulate its release. All these problems are avoided by collecting plasma. In agreement with Xu et al., ≈25% of the subjects had levels of Hsp60 below the limit of assay detection. However, a substantial proportion of this population, much higher than reported by Xu et al., contained significant levels of plasma Hsp60, with 27% containing >1000 ng/mL Hsp60. These are levels at which biological affects due to this protein, such as activation of myeloid cells and endothelial cells, would be expected.

Circulating Hsp60 was not associated with cardiovascular risk factors such as body mass index, blood pressure, and smoking status. We did record higher Hsp60 in people with an unfavourable lipid profile, as indexed by a low HDL cholesterol and a high total/HDL cholesterol ratio. No associations between antibodies to human Hsp60 and total or HDL cholesterol were recorded in cardiac patients. Xu et al. found that serum-soluble Hsp60 was associated with elevated LDL cholesterol but not with HDL cholesterol, blood pressure, or smoking.

The observed association between Hsp60 and levels of TNFα in the circulation is potentially important. TNFα is thought to play a direct role in atherogenesis and in the development of acute coronary syndromes. The association described here suggests that Hsp60 may be integral to the inflammatory processes implicated in CHD. In contrast, we found a negative association between Hsp60 and von Willebrand factor.

Psychosocial factors are causally involved in CHD, and meta-analyses indicate that low socioeconomic status, anxiety and depression, and social isolation are related to CHD. The presence and level of Hsp60 in plasma has not previously been associated with psychosocial factors. We found that the Hsp60 levels in plasma were greater in participants of lower socioeconomic status as defined by income, and that this effect was independent of relationships with lipids and inflammatory markers. Low income is related to increased CHD risk independently of standard cardiovascular risk factors.

People of lower socioeconomic status are more exposed to sources of chronic strain such as low job control, financial strain, and neighborhood stress. It is possible, therefore, that stress-related activation of inflammatory processes might stimulate the release of Hsp60 into the circulation.

The GHQ is a general measure of psychological distress and includes items related to anxiety and depression. Elevated Hsp60 was associated with psychological distress independently of other factors in women but not in men. The explanation for the sex difference is not clear. Equal proportions of men and women reported psychological distress, so underreporting by men is unlikely to be the explanation. It is possible that the self-reporting of emotional distress is differently regulated in men and women. The association in women provides further evidence that stress at the psychological and cellular level may affect Hsp60.

It is interesting that Hsp60 levels were higher in participants reporting low social support. This association did not survive multivariate analysis, suggesting that it coaggregates with psychosocial and biological factors. In the Whitehall II study, it has been found that social isolation is more common in people in lower grades of employment, so in the present investigation, low social support might have been one of the
manifestations of the psychosocial strain of lower socioeconomic status.

In summary, we have demonstrated that Hsp60 is found in the plasma of the majority of the individuals examined, with \( \approx 30\% \) of this population of civil servants containing biologically relevant levels of this stress protein >1000 ng/mL. The plasma levels of Hsp60 showed significant positive correlation with plasma TNF\( \alpha \) concentration, a negative association with von Willebrand factor, and a positive association with psychosocial factors, including income level, social isolation, and in women, psychological distress.

Acknowledgements
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References