Association between plasma levels of heat shock protein 60 and cardiovascular disease in patients with diabetes mellitus

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Aims Evidence is accumulating to support the hypothesis that the release of heat shock protein (Hsp)60 into the circulation is associated with the development of coronary heart disease (CHD). As diabetes is a risk factor for CHD, it was of interest to determine Hsp60 blood levels in a cross-sectional cohort of diabetic patients, some of whom had cardiovascular disease, and relate levels to relevant biochemical markers.

Methods and results A total of 855 patients with T1DM or T2DM, recruited as part of the UCL Diabetes and Cardiovascular disease Study (UDACS), were assayed for plasma levels of Hsp60. Immunoreactive Hsp60 was detected in 54% of the samples, with 26% having plasma levels $1 \mu g/mL$. Levels of Hsp60 were higher in Caucasians than in other ethnic groupings, with 56.5% of Caucasian subjects, 37.5% of African-Caribbean subjects, and 47.1% of Indian subjects having detectable levels ($P = 0.007$), and with a higher proportion of non-smokers having detectable Hsp60 levels than smokers (54.9 vs. 43.5%, $P = 0.01$). Of note was the finding of an association between higher mean plasma levels of Hsp60 in subjects with clinically manifest cardiovascular disease and those with a history of myocardial infarction having an adjusted odds ratio of having detectable Hsp60 of 2.17 (CI 1.26–3.73).

Conclusion This is the first report of circulating Hsp60 levels in diabetic patients, which suggests that this secreted mitochondrial cell stress protein may be playing an unexpected role in the cardiovascular pathology associated with diabetes.

KEYWORDS Coronary disease; Chaperonin; Risk factor; Hsp60; Myocardial infarction; UDACS

Introduction

Heat shock protein (Hsp)60 is a nuclear-encoded protein which functions as a major mitochondrial molecular chaperone. It exhibits significant homology with bacterial molecular chaperones, but in spite of this, the Hsp60 proteins of bacteria are normally powerful immunogens. Over the past decade, Wick et al. have generated experimental data to support the hypothesis that atherogenesis is driven by cross-reactive immunity to bacterial Hsp60 proteins. In this paradigm, various factors can cause the expression of host Hsp60 on the surface of vascular endothelial cells (VECs). This expression of an intracellular antigen generates an immunological response on the surface of the VEC layer, resulting in localized atherogenesis. Around 20 clinical studies have now been conducted in which titres of circulating antibodies against Hsp60 have been measured in individuals with atherosclerosis. These results suggest that anti-Hsp60 antibodies are a risk factor for coronary heart disease (CHD).

Most immunogens are simply molecules able to raise an immune response. However, Hsp60 is an unusual immunogen in that it has agonist activity and is able to activate immune cells and VECs. It is thus possible that this immune-activating capacity enhances the immunogenicity of Hsp60. Studies of heat shock proteins in samples of human blood cells led Pockley and co-workers to measure Hsp60 in normal human blood. Unexpectedly, this intracellular protein was found in the serum of normal individuals. This raised the question of whether circulating Hsp60 was a risk factor in atherosclerosis. Pockley et al. reported that blood Hsp60 levels are elevated in individuals with borderline hypertension, and Wick and co-workers, studying individuals participating in the Bruneck study
found that circulating Hsp60 levels correlated with arterial
intima-media thickness. In another study, this association
was also identified in a 17–18-year-old male cohort. We
have also recently reported that the presence of circulating
Hsp60 in healthy teenagers is associated with endothelial
dysfunction, as assessed by flow-mediated vasodilatation.
These findings suggest that the presence of Hsp60 in the cir-
culation may be associated with early vascular injury.

There is evidence that immunity to Hsp60 is important in
T1DM. This comes from the investigation of the non-obese
diabetic mouse which develops high titres of antibodies to
Hsp60 and individuals with T1DM who demonstrate T cell
responses to Hsp60. In addition, immunization of new-
onset T1DM patients with an Hsp60 peptide has been
reported to prevent disease progression. Given the
finding of a relationship between circulating Hsp60 and
atherosclerosis, we measured the circulating levels of
Hsp60 in patients with T1DM and T2DM and determined if
these were associated with other biochemical and/or clini-
cal parameters relevant to atherogenesis.

Methods

Subjects were recruited from the University College London
Diabetes and Cardiovascular Study (UDACS), described elsewhere. This is a cross-sectional sample designed to study the association
between common variants in inflammatory/metabolic genes and
biochemical risk factors implicated in cardiovascular disease (CVD)
in patients with diabetes. Briefly, this comprised 855 consecutive
subjects recruited from the diabetic clinic at the University
College London Hospital’s NHS Trust (UCLH) between the years
2001 and 2002. Of the 855 patients recruited, 17.2% had T1DM
and 82.8% had T2DM according to World Health Organization cri-
teria. No subjects requiring renal dialysis were recruited.
Ethical approval was obtained for this study from the UCLH Ethics
Committee. Subjects were categorized at recruitment by the pre-
ence/absence of clinically manifest CVD. CVD was recorded if a
patient had one or more of CHD, peripheral vascular disease
(PVD), or cerebrovascular disease (CBV). The presence of CHD
was recorded if any patient had positive coronary angiography or
angioplasty, coronary artery bypass, a positive cardiac thallium
scan or exercise tolerance test, or documented evidence of myocar-
dial infarction (MI) or symptomatic/treated angina. The presence
of PVD was recorded in any patient with absent peripheral pulses and
abnormal lower-limb Doppler pressures or an abnormal lower-limb
angiogram, previous angioplasty, or limb bypass graft. CBV was
recorded if a patient had been investigated for symptoms or signs
consistent with a cerebrovascular accident and had a brain com-
puted tomography scan showing any evidence of infarction
(diffuse/localized) or haemorrhage. Subjects who were asympto-
matic for CHD/CBV/PVD or who had negative investigations were
categorized as having no CVD.

Biochemical measurements

Plasma total antioxidant status

Plasma total antioxidant status (TAOS) was measured by a
photometric microassay described previously. The TAOS of
plasma samples was determined by the capacity to inhibit the
peroxidase-mediated formation of 2,2'-azino-bis-3-ethylbenzthiazoline-
6-sulfonic acid (ABTS·) radical. In the assay, the relative inhibition of
ABTS· formation in the presence of plasma is proportional to the
antioxidant capacity of the sample. Therefore, there are two arms to
the assay, a control arm and a test arm. In the control arm,
phosphate-buffered saline is used instead of plasma. The assay is
performed in a 96-well ELISA plate using 2.5 μL of plasma. The
difference in absorbance (control absorbance minus test
absorbance) divided by the control absorbance (expressed as a
percentage) was used to represent the percentage inhibition of
the reaction. Plasma TAOS is therefore inversely related to the
oxidative stress (the higher the oxidative stress, the lower the
TAOS). The inter-assay coefficient of variation (CV) was 14.1%,
and the intra-assay CV was 4.3%. As previously published, we
have found this to be an inexpensive and efficient marker of
plasma TAOS with a good correlation with plasma
F₂-isoprostanate.

Human Hsp60

Hsp60 was measured by two-site ELISA. Briefly, 96-well plates (Nunc
Maxisorp) were coated with mouse monoclonal anti-Hsp60 antibody
(clone LK-1, Stressgen, Victoria, Canada) at 0.5 μg/mL in PBS over-
night at 4°C. Plates were washed in wash buffer (0.5 mol/L NaCl,
2.5 mmol/L NaH₂PO₄, 7.5 mmol/L Na₂HPO₄, and 0.1% Tween 20),
and non-specific binding sites were blocked by incubation with 1%
bovine serum albumin (Sigma) in wash buffer for 2 h at room tempera-
ture. After washing, recombiant Hsp60 (Stressgen; 0–2500 ng/mL) or
dilutions of human plasma were added, and plates were incubated
for 2 h at 37°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 dilution
for 1 h at 37°C. Plates were washed and incubated with
poly-HRP-conjugated streptavidin (CLB) at 1/10 000 dilution for
30 min at 37°C. Binding of conjugated antibody was detected
using 1,2-phenylenediamine 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.

Other clinical assays

Assays of plasma glucose, Hba1c, total cholesterol, high density
lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) choles-
terol, triglyceride, creatinine, and C-reactive protein were assayed
using standard assay procedures in the Department of Clinical
Chemistry at the UCLH.

Statistical analysis

Associations with Hsp60 as a continuous variable were assessed using
Spearman rank correlation for continuous variables and by
Kruskal–Wallis test for categorical variables. The distribution of
Hsp60 was very skewed and was therefore further analysed by divid-
ing into groups above and below selected cut points (0, 1000, and
1000 ng/mL). Continuous variables were log-transformed before
analysis, where appropriate, and compared in those above and
below the cut points using two-sample t-tests (for normally or log-
normally distributed variables) or Mann–Whitney U tests. Differ-
ces in categorical variables were tested by χ² test or Fisher’s
exact test. Major endpoints were CVD, CHD (including MI), CBV,
and PVD. Median Hsp60 levels in those with and without events
were compared using Mann–Whitney U tests. The study was cross-
sectional with endpoints assessed at recruitment and was therefore
analysed as a case–control study with risk compared in those above
and below each cut point using a logistic regression model. Smoking
and ethnic group were found to be associated with hsp60 and were
therefore included as covariates in the model. Age and sex were
considered to be potential confounders and also included in the
model. Odds ratios (OR) and 95% CIs were obtained from the
model for both unadjusted associations and after adjustment for
age, sex, ethnic group, and smoking. A tobit regression model was
also fitted using log e (Hsp60 + 1) as a continuous variable. Results
from this model are presented as the ratio of hsp60 + 1 in cases
compared with controls. All tests were two sided. The significance
level was taken as P < 0.05, and no adjustments were made to
take account of multiple comparisons, because this has been
suggested to lead to more errors in interpretation.
should therefore be interpreted in the context of the number of tests conducted and require corroboration from other studies.

Results
The clinical characteristics of the participants in this study are summarized in Table 1. As expected, those with CVD were older, and a higher proportion were being treated with aspirin and statins. As a result, their mean plasma cholesterol levels were significantly lower than the non-CVD group.

Concentration of Hsp60 in plasma
A total of 855 plasma samples were assayed for immunoreactive Hsp60, with 54% (458/855) being positive for the presence of this protein. Levels of Hsp60 ranged from low nanograms per millilitre to milligrams per millilitre (Figure 1). The median level of Hsp60 was 27 ng/mL (interquartile range 0–1080). Further analysis revealed that 46% (397/855) of patients had levels of Hsp60 below the limit of assay sensitivity (1 ng/mL), 28% (240/855) had between 1 and 1000 ng/mL, and 26% (218/855) had >1 µg/mL (Figure 2). There was no statistical difference (P = 0.20, test for trend) in this distribution between patients with T1DM and those with T2DM (Figure 2). Overall, of those patients with detectable Hsp60, 7% had levels >100 µg/mL. The proportion of patients with T1DM or T2DM with Hsp60 levels >100 µg/mL was 9 and 6%, respectively. However, this difference was not statistically significant (P = 0.28).

Associations between plasma Hsp60 concentration and other biological variables
There was no association between the circulating concentrations of Hsp60 and age, weight, body mass index (BMI), blood pressure, or the concentration of plasma glucose, cholesterol, HDL, LDL, triglyceride, creatinine, or C-reactive protein. A proportion of this cohort was being treated with either aspirin and/or a statin, but there were no differences in the distribution of Hsp60 levels in patients taking these medications.

Relationship between ethnicity and circulating Hsp60 concentration
The patient cohort contained individuals from four different ethnic groups: Caucasian, African-Caribbean, south Indian and Oriental. A significantly higher proportion (P = 0.007) of Caucasian subjects, compared with the other ethnic groups, had detectable levels of Hsp60 and the range of Hsp60 concentrations in these Caucasian subjects was significantly greater (P = 0.01) than that in the other ethnic groups (Table 2).

Relationship between circulating Hsp60 concentration and smoking
An unexpected finding was that individuals who had never smoked, or were currently non-smokers, had significantly higher levels of circulating Hsp60 than individuals who currently were smokers. As shown in Table 3, 54.9% of non-smokers and ex-smokers compared with 43.3% of current smokers had detectable Hsp60 (P = 0.02). This was also reflected in the observation that 27% of non-smokers and ex-smokers had a circulating Hsp60 >1000 ng/mL compared with 18% of smokers, P = 0.04.

Circulating Hsp60 and cardiovascular events
A significantly higher proportion of patients with CVD had detectable circulating levels of Hsp60 compared with those without CVD (P = 0.04, Table 4). In addition, the proportion of subjects with detectable Hsp60 was higher in those with a history of MI compared with those without (70.2 vs. 52.0%, P = 0.004). In line with this, a significantly higher median level was also noted in those with MI compared with those without [185.3 (0–2619) vs. 21.5 (0–1006), P = 0.009]. The OR for MI was 2.29 (95% CI 1.26–3.73; P = 0.005) for those with detectable Hsp60. The tobit regression model confirmed the association with a ratio of 12.3 (2.0–76.7; P = 0.007). This effect was maintained after adjustment for age, sex, ethnic group, and smoking (Table 4).

<table>
<thead>
<tr>
<th>Variable</th>
<th>No CVD (n = 607)</th>
<th>CVD (n = 241)</th>
<th>P-value</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>59.9 (13.5)</td>
<td>68.3 (9.8)</td>
<td>&lt;0.0001</td>
<td>62.3 (13.1)</td>
</tr>
<tr>
<td>Sex, %male</td>
<td>56.5 (343)</td>
<td>69.3 (167)</td>
<td>0.001</td>
<td>60.0 (511)</td>
</tr>
<tr>
<td>BMIa</td>
<td>28.1 (5.3)</td>
<td>28.4 (4.7)</td>
<td>0.52</td>
<td>28.2 (5.2)</td>
</tr>
<tr>
<td>SBP (lying)a</td>
<td>139.2 (17.5)</td>
<td>138.8 (19.9)</td>
<td>0.80</td>
<td>139.1 (18.1)</td>
</tr>
<tr>
<td>DBP (lying)</td>
<td>80.1 (10.7)</td>
<td>77.3 (10.0)</td>
<td>0.001</td>
<td>79.2 (10.6)</td>
</tr>
<tr>
<td>Glucosea</td>
<td>9.84 (4.93)</td>
<td>9.66 (4.36)</td>
<td>0.61</td>
<td>9.79 (4.76)</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>5.16 (0.99)</td>
<td>4.84 (1.11)</td>
<td>&lt;0.0001</td>
<td>5.07 (1.03)</td>
</tr>
<tr>
<td>HDLa</td>
<td>1.36 (0.43)</td>
<td>1.24 (0.40)</td>
<td>&lt;0.0001</td>
<td>1.33 (0.42)</td>
</tr>
<tr>
<td>LDL</td>
<td>2.89 (0.92)</td>
<td>2.57 (0.96)</td>
<td>&lt;0.0001</td>
<td>2.80 (0.94)</td>
</tr>
<tr>
<td>Triglyceridea</td>
<td>1.66 (1.00)</td>
<td>1.90 (1.08)</td>
<td>0.003</td>
<td>1.73 (1.03)</td>
</tr>
<tr>
<td>Smoking, %current</td>
<td>15.9 (95)</td>
<td>13.6 (32)</td>
<td>0.40</td>
<td>15.2 (127)</td>
</tr>
<tr>
<td>Aspirin use, %yes</td>
<td>33.6 (203)</td>
<td>77.4 (185)</td>
<td>&lt;0.0001</td>
<td>46.0 (388)</td>
</tr>
<tr>
<td>Statin use, %yes</td>
<td>16.6 (100)</td>
<td>52.9 (126)</td>
<td>&lt;0.0001</td>
<td>26.9 (226)</td>
</tr>
<tr>
<td>Diabetes type, %type 2</td>
<td>77.9 (472)</td>
<td>95.0 (229)</td>
<td>&lt;0.0001</td>
<td>82.8 (701)</td>
</tr>
</tbody>
</table>

*Log-transformed before analysis.
Discussion

The data are accumulating to support the fact that human Hsp60 is a pro-inflammatory protein, but such pro-inflammatory activity will remain unexpressed while the Hsp60 is sequestered in cells. The finding of Hsp60 free in the circulation was thus unexpected, and this circulating Hsp60 may be activating target cells and promoting the process of atherogenesis, as evidenced from several studies. The simplest conclusion which can be made from these data is that the release of Hsp60 into the circulation is an early risk factor in atherogenesis. Because diabetes mellitus is a well-established risk factor for CVD, and there is good evidence that immunity to Hsp60 is involved in type I diabetes, it could be predicted that the concentrations of Hsp60 in patients with T1DM and T2DM will be elevated. This hypothesis was confirmed in the patients in the UDACS. Overall, 54% of the subjects had detectable Hsp60, which ranged from low nanograms per millilitre to, in a few samples, milligrams per millilitre. In line with the data from the Bruneck study, and from civil servants participating in the Whitehall study, the distribution of Hsp60 levels suggests that there are three sub-populations within the diabetics examined, with 46% of subjects having no measurable Hsp60, 28% having between 1 and 1000 ng/mL, and 26% having >1 µg/mL. In the Bruneck study, 33% had no circulating Hsp60, but only 5% had levels >1 µg/mL. However, in the Whitehall study, 22% had undetectable Hsp60 and 20% of the sample had >1 µg/mL of Hsp60 in the circulation. The patients within UDACS show the highest levels of Hsp60 that we have yet observed, with 7% of the subjects exhibiting Hsp60 levels >100 µg/mL. At this plasma concentration, Hsp60 is biologically active, being able to stimulate myeloid cell to produce pro-inflammatory mediators and VECs to express vascular adhesion proteins which could directly contribute to the pathogenesis of atherosclerosis. Compared with the Whitehall study (carried out with the identical assay), the mean Hsp60 concentration in this study was much higher (36 527 vs. 5798 ng/mL). However, the median of 27 ng/mL was significantly lower than 110 ng/mL in the Whitehall study (P = 0.0004). This is due to higher percentage of non-detectable Hsp60 samples, with an interquartile range of 0–1080 compared with 13–1589 from the Whitehall study.

Table 2  Plasma Hsp60 levels in subjects of different ethnicity

<table>
<thead>
<tr>
<th>Ethnic group</th>
<th>Hsp60 [median (IQR)] ng/mL</th>
<th>P-valuea</th>
<th>Percentage of patients with Hsp60 &gt; 0</th>
<th>P-valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td>African-Caribbean (n = 67)</td>
<td>0 (0–382)</td>
<td>0.01</td>
<td>37.3</td>
<td>0.007</td>
</tr>
<tr>
<td>Caucasian (n = 665)</td>
<td>41.6 (1–1270)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indian (n = 102)</td>
<td>0 (0–349)</td>
<td></td>
<td>47.1</td>
<td></td>
</tr>
<tr>
<td>Oriental</td>
<td>0 (0–27)</td>
<td></td>
<td>41.2</td>
<td></td>
</tr>
</tbody>
</table>

*aKruskal–Wallis.  
*bχ².

Table 3  Relationship between smoking status and Hsp60 levels

<table>
<thead>
<tr>
<th>Smoking status</th>
<th>Hsp60 concentration [median (IQR)]</th>
<th>P-valuea</th>
<th>Percentage of patients with Hsp60 &gt; 0</th>
<th>P-valueb</th>
<th>Percentage of patients with Hsp60 &gt; 1000</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-smokers</td>
<td>36.9 (0–1034)</td>
<td>0.01</td>
<td>54.9</td>
<td>0.02</td>
<td>26.8</td>
<td>0.04</td>
</tr>
<tr>
<td>Smokers</td>
<td>0 (0–389)</td>
<td></td>
<td></td>
<td></td>
<td>43.3</td>
<td></td>
</tr>
</tbody>
</table>

*aKruskal–Wallis.  
*bχ².
Asian Indians have a higher prevalence of diabetes. It was observed a strong correlation between psychological stress and circulating Hsp60 levels. One plausible explanation might be that if a subject is a smoker and diabetic, the plasma levels of Hsp60 might be higher, but the increased mortality associated with the combination of these risk factors (i.e. smoking and diabetes) results in an observed low mean plasma level in the surviving smokers. This is speculative and needs to be re-assessed in a prospective study.

Previous studies of Hsp60 levels have strongly indicated a relationship between blood levels of this protein and pathology of the vasculature. In the UDACS subjects, we observed that a significantly higher proportion of patients with CVD had measurable levels of Hsp60, compared with those who had not been diagnosed with CVD. This was most apparent among those patients with MI, with 70% having detectable levels compared with 52% of the no-MI subjects. Animal studies have shown over-expression of Hsp60 in infarcted rodent heart tissue which could be released from heart into the circulation several hours after heart ischaemia and cause elevated levels in post-MI subjects. However, anti-Hsp60 antibody titres have been reported to decrease actively after MI, possibly because the large amount of Hsp60 released could form immune complexes with pre-existing anti-Hsp60 antibodies. The observation in the current study cannot determine whether Hsp60 may be cause or effect of MI or CVD, but clearly, this is speculative, and no molecular mechanism can be suggested by this cross-sectional data.

One limitation of this study was that the subjects within UDACS were taking a mixture of oral hypoglycaemic agents, insulin, antihypertensive agents, and lipid-lowering therapy. It was not possible to adjust or stratify for all of these therapies in the analysis, as this would considerably reduce power. Furthermore, this is a cross-sectional sample and hence does not provide information with respect to causality, which might be gained from a prospective study. Another limitation is that plasma TAOS is not a highly specific measure of plasma oxidative stress. However, for large studies such as UDACS, it is a practical and an inexpensive assay. By comparison, more specific assays of plasma oxidation (e.g. plasma F2-isoprostanes) are time-consuming and expensive, but we have previously observed a strong correlation between plasma TAOS and esterified F2-isoprostane in a sub-sample from UDACS. We have also previously reported an association between elevated baseline plasma TAOS and higher 10-year prospective CHD-risk, supporting the utility and biological relevance of this assay.

It is known that certain ethnic groups are more prone to diabetes than others, and that African-Americans and Asian Indians have a higher prevalence of diabetes. It was therefore intriguing to find that a significantly higher proportion of the Caucasians in this group of patients had detectable Hsp60 than did the patients of African-Caribbean, Indian, or Oriental origin. Although numbers are small, this is the first indication that there may be ethnic differences in the circulating levels of a heat shock protein, and this preliminary finding needs to be followed up.

Smoking, together with high levels of cholesterol, hypertension, and diabetes, is one of the major and classical risk factors for atherosclerosis. Therefore it was surprising that individuals who had never smoked, or were currently not smoking, had significantly higher levels of plasma Hsp60 than did current smokers; this was also seen as a significantly higher proportion of non-smokers with plasma levels >1000 ng/mL. In a previous study, we found a direct correlation between psychological stress and circulating Hsp60 levels. One plausible explanation might be that the relaxant effects of smoking could, by an undefined mechanism, result in lower levels of this stress protein. Alternatively, this might arise owing to survival bias, such that if a subject is a smoker and diabetic, the plasma levels of Hsp60 might be higher, but the increased mortality

<table>
<thead>
<tr>
<th>Event</th>
<th>Median Hsp60 (IQR)</th>
<th>Proportion with detectable Hsp60 percentage (n)</th>
<th>Odds (95% CI) of detectable Hsp60</th>
<th>Ratio (hsp60 + 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHD/stroke/PVD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Event</td>
<td>60.8 (0–888)</td>
<td>58.9 (142)</td>
<td>1.51 (1.09–2.10)</td>
<td>3.31 (1.00–11.03)</td>
</tr>
<tr>
<td>No event</td>
<td>19.7 (0–1119)</td>
<td>51.2 (311)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>P</td>
<td>0.16</td>
<td>0.04</td>
<td>0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>MI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Event</td>
<td>185.3 (0–2619)</td>
<td>70.2 (47)</td>
<td>2.19 (1.26–3.81)</td>
<td>12.4 (2.0–77.5)</td>
</tr>
<tr>
<td>No event</td>
<td>21.5 (0–1006)</td>
<td>52 (406)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>P</td>
<td>0.009</td>
<td>0.004</td>
<td>0.006</td>
<td>0.007</td>
</tr>
</tbody>
</table>

*Adjusted for age, sex, ethnic group, and smoking.

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