Increased titres of anti-human heat shock protein 60 predict an adverse one year prognosis in patients with acute cardiac chest pain

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Objective: To assess whether antibodies to human heat shock protein 60 (anti-huhsp60) or to mycobacterial heat shock protein 65 (anti-mhsp65) predict an adverse one year prognosis in patients admitted with acute cardiac chest pain.

Design: Prospective observational study.

Setting: Teaching hospital.

Patients: 588 consecutive emergency admissions of patients with acute chest pain of suspected cardiac origin.

Main outcome measures: Anti-huhsp60 and anti-mhsp65 titres were assayed on samples drawn on the morning after admission. The end points after discharge were coronary heart disease death, non-fatal myocardial infarction, coronary artery bypass grafting, percutaneous transluminal coronary angioplasty, angiogram, or readmission with further cardiac ischaemic chest pain.

Results: During follow up after discharge (mean of 304 days, range 1–788 days), 277 patients had at least one of the study outcomes. Patients with increased titres of anti-huhsp60 had an adverse prognosis (hazard ratio 1.56 (95% confidence interval 1.09 to 2.23) comparing highest versus lowest quartiles, p = 0.015). Anti-mhsp65 titres were not predictive.

Conclusions: Patients admitted with acute cardiac chest pain and increased titres of anti-huhsp60 had an adverse one year prognosis.

The inflammatory state of atherosclerosis is important in determining plaque stability. Increased C reactive protein (CRP) concentrations have important prognostic implications and interest has turned to other aspects of inflammation including antibodies to heat shock protein (hsp) 60/65. The hypothesis linking hsp60/65 with atherosclerosis originated from the work of Xu and colleagues. Xu et al published in 1993 the first clinical study relating autoimmunity to hsp60/65 with atherosclerosis. They showed that an increased antimycobacterial hsp65 (anti-mhsp65) titre was independently associated with the presence of carotid atherosclerosis. Subsequently some studies have found a positive association between anti-msp65 and anti-human hsp60 (anti-huhsp60) titres and prevalent coronary atherosclerosis. Four large prospective studies have shown significant associations of anti-huhsp60 or anti-mhsp65 titre with development of carotid atherosclerosis or with clinical events.

The patients in these prospective studies were all stable at inclusion. This study is the first to assess whether increased titres of anti-huhsp60 have prognostic significance in patients presenting with unstable coronary heart disease (CHD), where the atherosclerotic inflammatory state may be importantly different from the stable baseline state.

METHODS

Patients
All patients admitted to the emergency department with acute chest pain of suspected cardiac ischaemic origin were eligible for inclusion. Patients were assessed on the first morning after admission to hospital by patient history, clinical examination, and available 12 lead ECGs. ECG abnormalities or other evidence of coronary artery disease was not required for enrolment. The only exclusion criteria were non-ischaemic chest pain; definite ST elevation acute myocardial infarction (MI); poor one year non-cardiac prognosis; and lack of informed consent. All patient management decisions were made solely by the treating physician. The protocol was approved by the hospital’s ethics committee.

Clinical assessment
All patients were seen by a single investigator (L E V), who obtained written informed consent from all patients. A standardised history and physical examination were performed and the results were entered on to case record forms.

Electrocardiography
One of the investigators (LEV) evaluated the admission ECG. The ECG was categorised as follows: (a) previous myocardial infarction (MI, pathological Q waves, an otherwise unexplained positive R wave in lead V1 indicative of established posterior MI, or left bundle branch block); (b) significant ST-T wave abnormality (> 1 mm ST segment deviation in two or more contiguous leads, or > 1 mm T wave inversion in two or more contiguous leads with predominantly positive QRS complexes; no previous MI); (c) minor abnormality (any ECG abnormality not satisfying either of the above criteria; no previous MI or significant ST-T wave abnormality); and (d) normal. All subsequent ECGs recorded during the admission stay were analysed for evidence of new MI and significant

Abbreviations:
BSA, bovine serum albumin; CABG, coronary artery bypass grafting; CHD, coronary heart disease; CI, confidence interval; CRP, C reactive protein; ELISA, enzyme linked immunosorbent assay; hsp, heat shock protein; huhsp, human heat shock protein; mhsp, mycobacterial heat shock protein; MI, myocardial infarction; PBS, phosphate buffered saline; PTC, percutaneous transluminal coronary angioplasty
new ST-T wave changes as defined by category (b) above when compared with the most recent recording.

Assays
On the first morning after admission, 20 ml of venous blood was obtained. The blood was centrifuged for 30 minutes at 2000 g and 4°C. Plasma was collected and stored at −70°C until use. Anti-mhsp65 and anti-huhsp60 titres were subsequently measured. The first of these assays is an enzyme linked immunosorbent assay (ELISA) based on a modification of the method of Xu et al.17 and we have previously validated and published the technique. Briefly, microtitre plates were coated with 1 μg/ml of recombinant mhsp65 in 100 μl phosphate buffered saline (PBS) in each well at 4°C overnight. The plates were then washed with a 0–0.1% Tween solution in PBS and blocked with 200 μl 0–1% bovine serum albumin (BSA) in PBS (PBS-BSA) at room temperature for one hour. The plates were washed again and then incubated with 100 μl of serum samples diluted 1:400 with PBS-BSA. After a further wash in PBS-Tween, the plates were incubated with horseradish peroxidase conjugated rabbit anti-human IgG (Dakopatts, Glostrup, Denmark) diluted 1:3000 with PBS-BSA. This was left at room temperature for one hour and the plates were washed with PBS-Tween again. Colour was developed with 5-phenylenediamine and the reaction was stopped with 4 mol/l H2SO4. The standard consisted of caprylic acid purified IgG from a patient with a high anti-mhsp65 concentration. The mean absorbance was calculated for each test sample and the serial dilutions of the standard. The unknown values for each test sample were read against the standard curve and values expressed as U/l. The anti-huhsp60 assay was developed along similar lines. Recombinant huhsp60 was obtained from StressGen Biotechnologies Corp (Victoria, British Columbia, Canada).

CRP was assayed by an in-house ELISA with rabbit anti-human CRP antibodies on Nunc microtitre plates. The sensitivity of this assay was 0.001 mg/l. Troponin T was determined by a chemiluminescent sandwich immunoassay and the sensitivity of this assay was 0.01 μg/l. Physicians were blinded to the results all assays.

Follow up
After discharge, the patients were followed up by review of their hospital case records and directly by telephone or letter. General practitioners were contacted by letter when direct patient contact was unsuccessful. Case records from other hospitals or primary care were reviewed in cases where patients had possible end points in other hospitals or the community.

End point
The hard end point was defined as time to the first of death caused by CHD, including sudden death, or non-fatal MI. The hard plus soft end point was time to the first of CHD death, non-fatal MI, coronary artery bypass grafting (CABG), percutaneous transluminal coronary angioplasty (PTCA), angiogram, or readmission to hospital with chest pain. Angiograms, CABGs, and PTCA already arranged before the index admission were not counted as end points. Angiograms performed or arranged during the index admission also were not counted.

Statistical analysis
The characteristics of the cohort were summarised by the number (%) of patients for categorical covariates and mean (SD) or median (interquartile range) for continuous covariates (table 1 lists the covariates of interest). Anti-huhsp60, anti-mhsp65, and CRP were log transformed for analysis. Spearman rank correlations were calculated between the continuous covariates and the antibody titres, and two-sample t tests were used to compared mean titres of binary variates. The individual relation of each covariate to both the hard and the hard plus soft end points separately was assessed by univariate Cox proportional hazards models, with hazard ratios (and 95% confidence intervals (CIs) with associated Wald p values) calculated for the stated increment in the covariate (for example, an increase of five years in age, or the presence or absence of diabetes). The quartiles of each antibody titre and a binary indicator of high (> 90th centile) antibody titre were also investigated. Kaplan-Meier survival plots stratified by the anti-huhsp60 titre quartiles were drawn. Multivariate Cox proportional hazards models were used to adjust any effect seen univariately for the anti-huhsp60 titres (both as a continuous covariate and in quartiles) for age, history of hypertension, diabetes, smoking, and log(CRP). All analyses were performed with SAS 8.2 for Windows (SAS Institute, Cary, North Carolina, USA). No adjustment has been made for multiple testing.

RESULTS
Patients
A total of 710 consecutive patients were enrolled from a single centre. Of these, 33 were subsequently found to have had an MI (on the basis of development of new pathological Q Waves) at the time of index admission and were therefore excluded, leaving 677 patients. Also samples from 89 patients were lost, assumed to be missing at random, leaving a cohort of 588 patients with analysable data. Of these 588 patients, 13 did not have an anti-huhsp60 measurement and 10 did not have an anti-mhsp65 measurement. Table 1 summarises baseline clinical characteristics of the 588 patients.

Follow up and clinical outcome
Only one patient was lost to follow up after hospital discharge. During follow up (mean of 304 days, range 1–788 days), 277 patients had any study outcome end point, of
whom 71 had a hard end point first (CHD death or non-fatal MI) and 206 a soft end point initially (PTCA or CABG or angiography or readmission to hospital for chest pain). The numbers of patients experiencing each component of the composite hard plus soft outcome were as follows: 179 were readmitted for chest pain; 81 underwent angiography; 32 underwent PTCA; 32 underwent CABG; 54 died of CHD causes; 22 had a non-fatal MI.

**Antibody titres and baseline variables**

The Spearman rank correlation between anti-huhsp60 and anti-mhsp65 titre was 0.2. Both antibodies were weakly positively correlated with age \(r = 0.20\) and with CRP \(r = 0.15\). There was no correlation with white cell count or with creatinine. There was no significant relation between either of the antibody titres and any of the categorical baseline variables in table 1. In particular it should be noted that there was no association between antibody titres and history of atherosclerosis (either CHD or stroke). There were trends for patients taking long term aspirin to have lower titres of both anti-huhsp60 \(p = 0.07\) and anti-mhsp65 \(p = 0.1\).

Table 2 shows univariate hazard ratios (with 95% CIs and \(p\) values) for other risk factors.

**Anti-huhsp60 titres and prognosis**

Table 3 details univariate and multivariate hazard ratios (with 95% CIs and \(p\) values) for anti-huhsp60 titre. In univariate analysis there was a trend for patients with increased titres of anti-huhsp60 to have an increased risk of a hard end point (CHD death or non-fatal MI); hazard ratio 1.82, 95% CI 0.92 to 3.62 comparing highest versus lowest quartiles, \(p = 0.087\). We found a graded increase in risk for the hard end point, with hazard ratios increased by about 80% in the top two quartiles, but overall these differences did not achieve significance.

Patients with raised concentrations of anti-huhsp60 did have a significantly increased risk for the composite hard and soft end point. This risk persisted after adjustment for covariates that have been previously shown to influence anti-huhsp60 titre (age, hypertension, diabetes, and smoking) and the best studied inflammatory predictor, CRP. The hazard ratio was 1.56 (95% CI 1.09 to 2.23) comparing highest versus lowest quartiles \((p = 0.015)\). All patients above the first quartile \(>= 16\) U/l had an increased risk, with a hazard ratio about 50% higher. Figures 1 and 2 shows Kaplan-Meier survival curves for anti-huhsp60 titre (Q1 to Q4).
The first quartile (≥ 16 U/l) had an increased risk, with a hazard ratio about 50% higher. A more graded increase in risk was observed for the hard end point, with hazard ratios increased by about 80% in the top two quartiles. Overall, these differences did not achieve significance, in part due to the lower number of hard end points. These patterns were still in evidence after adjustment for the established cardiovascular risk factors of age, hypertension, diabetes, and smoking. Furthermore, the risk was independent of the best studied inflammatory predictor, CRP. Anti-mhsp65 titres were not predictive. Also there was no significant association between anti-huhsp60 and outcome in the troponin positive group. However, we suspect this is simply due to inadequate sample size, as there were only 120 combined end points in this group.

Evidences is accumulating that the inflammatory-immunological state of atherosclerosis is important in determining vulnerability to plaque rupture. In some cohorts, a higher concentration of CRP (a non-specific marker of inflammation) is a risk factor for progression of atherosclerosis to MI. Thus, it has been suggested that the biological state of a coronary lesion may be a more important determinant of the clinical outcome than, for example, the degree of stenosis. Interest has turned to other aspects of inflammation including antibodies to hsps60/65. The hsps60/65 family comprises huhsp60, mhsp65, the GroEL protein of Escherichia coli, huhsp60 of Chlamydia pneumoniae, hsps62 of Helicobacter pylori, and many others. Autoimmunity to huhsp60 provoked by infection with C pneumoniae and perhaps other microorganisms has been postulated as one explanation for the observed associations between these infections and atherosclerosis.

Four published prospective studies have examined the prognostic implications of anti-huhsp60 and anti-mhsp65. The first of these found that increased anti-mhsp 65 titres predicted mortality in a cohort with carotid atherosclerosis. The second found that anti-mhsp65 titres predicted progression of carotid atherosclerosis over five years. Neither study examined anti-huhsp60 titre.

The third study examined both anti-mhsp65 and anti-huhsp60 titres in a nested case-control study of 386 patients with cardiovascular events (MI, cerebrovascular accident, cardiovascular death) and the same number of age and sex matched controls over a mean follow up of 4.5 years. Median serum concentrations of anti-mhsp65 antibodies was significantly higher in the patients than in the controls, and high titres (≥ 90th centile) of anti-mhsp65 antibodies

| Table 4 | Univariate and multivariate (adjusted for covariates listed in table 2) hazard ratios for various characterisations of the anti-mhsp65 titre for the end points |
|---------|-------------------------------------------------------------|-------------------------------------------------------------|

<table>
<thead>
<tr>
<th>Covariate</th>
<th>HR (95% CI)</th>
<th>p value</th>
<th>HR (95% CI)</th>
<th>p value</th>
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<tbody>
<tr>
<td>Hard end point</td>
<td></td>
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<tr>
<td>Log (anti-mhsp65)</td>
<td>1.10 (0.86 to 1.40)</td>
<td>0.47</td>
<td>0.95 (0.74 to 1.23)</td>
<td>0.70</td>
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<tr>
<td>≥90th centile anti-mhsp65</td>
<td>1.31 (0.65 to 2.64)</td>
<td>0.45</td>
<td>1.02 (0.50 to 2.07)</td>
<td>0.97</td>
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<tr>
<td>Anti-mhsp65 quartiles (U/l)</td>
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<tr>
<td>&lt;9</td>
<td>1.00</td>
<td>NA</td>
<td>1.00</td>
<td>NA</td>
</tr>
<tr>
<td>≥9–&lt;17</td>
<td>1.61 (0.81 to 3.22)</td>
<td>0.18</td>
<td>1.48 (0.74 to 2.99)</td>
<td>0.27</td>
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<tr>
<td>≥17–&lt;31</td>
<td>1.52 (0.75 to 3.11)</td>
<td>0.25</td>
<td>1.23 (0.60 to 2.54)</td>
<td>0.57</td>
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<tr>
<td>≥31</td>
<td>1.36 (0.69 to 2.78)</td>
<td>0.40</td>
<td>0.95 (0.46 to 1.97)</td>
<td>0.90</td>
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<tr>
<td>Overall p value</td>
<td>0.57</td>
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<td>0.52</td>
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<tr>
<td>Hard plus soft end point</td>
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<tr>
<td>Log (anti-mhsp65)</td>
<td>0.97 (0.86 to 1.10)</td>
<td>0.63</td>
<td>0.97 (0.86 to 1.11)</td>
<td>0.68</td>
</tr>
<tr>
<td>≥90th centile anti-mhsp65</td>
<td>0.87 (0.58 to 1.32)</td>
<td>0.52</td>
<td>0.87 (0.57 to 1.31)</td>
<td>0.50</td>
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<tr>
<td>Anti-mhsp65 quartiles (U/l)</td>
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<tr>
<td>&lt;9</td>
<td>1.00</td>
<td>NA</td>
<td>1.00</td>
<td>NA</td>
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<tr>
<td>≥9–&lt;17</td>
<td>1.06 (0.77 to 1.46)</td>
<td>0.74</td>
<td>1.03 (0.74 to 1.43)</td>
<td>0.87</td>
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<tr>
<td>≥17–&lt;31</td>
<td>0.76 (0.53 to 1.07)</td>
<td>0.12</td>
<td>0.76 (0.53 to 1.08)</td>
<td>0.12</td>
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<tr>
<td>≥31</td>
<td>0.94 (0.67 to 1.30)</td>
<td>0.69</td>
<td>0.94 (0.67 to 1.32)</td>
<td>0.74</td>
</tr>
<tr>
<td>Overall p value</td>
<td>0.27</td>
<td></td>
<td>0.33</td>
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were found significantly more often among patients than among controls (13.2% v 6.6%, \( p = 0.008 \)).\(^9\) Median titres of anti-huhsp60 were similar in patients and controls.

The fourth study\(^9\) examined separately titres of IgA and IgG antibodies to huhsp60 in a nested case–control study. IgA but not IgG huhsp60 titres were a significant risk factor for coronary events (odds ratio 2.0, 5% CI 1.1 to 3.6, when the fourth and first quartiles were compared). Further, they showed that the combination of an increased IgA anti-huhsp60 titre and increased CRP and increased C pneumoniae IgA antibody titre conferred an odds ratio of 3.0 (95% CI 1.8 to 14.2).\(^9\)

There are several possible explanations for the contrast in results between our study and other prospective studies. Most important, the patient populations were quite different. Our cohort was unique in being recruited when they were unstable, when the atherosclerotic inflammatory state may be postulated to be quite different from the stable baseline state in the four other studies. There is also some evidence that acute ischaemia alters the expression of huhsp60,\(^9\) which is likely to influence anti-huhsp60 titre. Thus, our study is very importantly different from the other studies in this regard. There was one other minor difference between our cohort and that of Veres et al\(^9\) in the proportion of patients who had had a previous MI. In our group only 19.9% had had a previous MI compared with 66.5% of patients in their study.\(^9\) Previous MI has been suggested to result in an important decrease in serum concentration of anti-huhsp60 antibodies.\(^9\) It seems likely, therefore, that titres of anti-hsp60 and the anti-hsp60-hsp60 reaction may have differing implications or effects in the three major stages of CHD (that is, chronic stable angina, unstable angina, and in the days and weeks after acute MI.) Stollberger and Finsterer noted the need for further study of this effect in the three stages in their editorial in The Lancet.\(^9\) Our study is the first to confirm the findings of Veres et al\(^9\) that anti-mhsp65 and anti-huhsp 60 titres are only weakly correlated. This is perhaps surprising because, like all hsp families, hsp60/65 is highly conserved between species—for example, mhps65 is 75% homologous with huhsp60.\(^9\) The weak correlation in titres suggests that anti-mhsp65 titres cannot be used as a surrogate for anti-huhsp60 titres and that future human studies should focus on the immune response to huhsp60. In our study there was only a minor correlation between CRP titre and huhsp60 titre. To our knowledge only Veres et al\(^9\) have performed a similar analysis and they found no correlation. Furthermore, in our study the relation between anti-huhsp60 titre and subsequent events was unchanged after correction for CRP. This all suggests that, although CRP and anti-huhsp60 titres are both inflammatory markers, they influence atherosclerotic risk through differing mechanisms.

There were interesting trends for patients taking long term aspirin to have lower titres of both anti-huhsp60 (\( p = 0.07 \)) and anti-mhsp65 (\( p = 0.1 \)). This has not previously been examined. There are similar data for CRP and aspirin. Low dose aspirin treatment reduces serum CRP concentrations\(^1\) in patients with but not in those without coronary artery disease.\(^2\) \(^3\) \(^9\) In men, low dose aspirin appears to have its greatest protective effect against MI when serum CRP concentrations are relatively high.\(^9\) These observations raise the possibility that low dose aspirin may actually protect against MI by reducing inflammation.

In conclusion, patients admitted with acute cardiac chest pain and increased titres of anti-huhsp60 had an adverse one year prognosis independent of other cardiovascular risk factors (age, hypertension, diabetes, and smoking). Furthermore, the risk was independent of the best studied inflammatory predictor, CRP. Anti-mhsp65 titres were not predictive.

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The work was performed in the Department of Medical Cardiology, Royal Infirmary, Scotland and the Department of Medicine and Therapeutics, University of Glasgow, Scotland.

REFERENCES


Fibrin thrombus causing myocardial infarction in a patient with patent coronary arteries

A 47 year old woman, without coronary artery disease risk factors, first noted severe chest tightness as she was hurrying to catch the subway (underground). Acute inferior wall ST segment elevation myocardial infarction was diagnosed.

Emergent coronary angiography revealed one vessel disease. The right coronary artery (RCA), the infarct related artery, was occluded with thrombus containing lesions over the middle portion (panel A, arrow). An Export aspiration catheter was advanced into the RCA for thrombosuction. However, poor coronary flow was still noted due to ineffectiveness of removing large visible thrombi in the RCA. Balloon angioplasty was performed later without success, too. Since thrombosuction was considered to be able to relieve the thrombus burden rapidly, the guiding catheter was deeply advanced to the lesion (panel B) using the balloon catheter as an anchor. A large thrombus was aspirated. The aspirated thrombus, size 10 x 5 x 3 mm, white (panel D) was found to match the filling defect on the angiography. The final angiography revealed an excellent result (panel C) with a TIMI 3 flow. The histopathological examination showed that the aspirated thrombus was composed of fibrin material and few blood cells (panel E). The patient’s hospital course was smooth and she was discharged four days later.

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