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

D H Birnie, L E Vickers, W S Hills, J Norrie, S M Cobbe

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.1–6

Xu et al7 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.2 Subsequently some studies have found a positive association between anti-mp657–8 and anti-human hsp60 (anti-huhsp60)9–11 titres and prevalent coronary atherosclerosis. Four large prospective studies12–15 have shown significant associations of anti-huhsp60 or anti-mhsp65 titre with development of carotid atherosclerosis16 or with clinical events.12–15

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; PTCA, 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.16 and we have previously validated and published the technique.17 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 0–01% 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 1-phenylenediamine and the reaction was stopped with 4 mol/l H2SO4. The standard consisted of caprylic acid purified lgG 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 is 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), angio gram, or readmission to hospital with chest pain. Angiograms, CABGs, and PTCAFs 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 compare mean titres of binary variables. 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.

**Table 3** Details univariate and multivariate 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 \( (\geq 16 \text{ U/l}) \) had an increased risk, with a hazard ratio about 50% higher. Figures 1 and 2 shows Kaplan-Meier survival plots of time to first hard end point by anti-human heat shock protein 60 (anti-huhsp60) quartiles (Q1 to Q4).

**Figure 1** Kaplan-Meier survival plot of time to first hard end point by anti-human heat shock protein 60 (anti-huhsp60) quartiles (Q1 to Q4).
Anti-huhsp60 in acute cardiac chest pain

Figure 2 Kaplan-Meier survival plot of time to first hard plus soft end point by anti-huhsp60 quartiles (Q1 to Q4).

survival curves for anti-huhsp60 in quartiles for the hard end point and the combined hard plus soft end point.

Anti-mhsp65 titres and prognosis
Table 4 details the anti-mhsp65 data. There was no association between any of the characterisations of the anti-mhsp65 titre and either the hard end point or the composite end point.

Anti-huhsp60 titre and prognosis in the troponin positive group
Troponin T results from day 1 of the index admission were available for 526 patients, of whom 192 (37%) were troponin positive (> 0.01 μg/l). For the quartiles of anti-huhsp60 34%, 30%, 39% and 43%, respectively, were troponin positive. Refitting the same Cox models (as detailed above) to just the patients who were troponin positive showed no significant association between anti-huhsp60 and outcome (either hard or hard plus soft).

DISCUSSION
Our study indicates that patients admitted with acute cardiac chest pain and increased titres of anti-huhsp60 had an adverse one year prognosis for the hard plus soft outcome of CHD death or non-fatal MI plus angiography, PTCA, CABG, or readmission for cardiac chest pain. All patients above 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.

Evidence 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 hsp60/65. The hsp60/65 family comprises huhsp60, mhsp65, the GroEL protein of Escherichia coli, huhsp60 of Chlamydia pneumoniae, hsp62 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 |
| Covariate | Univariate | Multivariate | |
| Hard end point | | | |
| Log (anti-mhsp65) | | | |
| ≥90th centile anti-mhsp65 | | | |
| <9 | 1.61 (0.81 to 3.22) | 0.18 | |
| ≥9–<17 | 1.21 (0.75 to 2.01) | 0.26 | |
| ≥17–<31 | 1.36 (0.69 to 2.78) | 0.45 | |
| ≥31 | 0.57 | 0.52 | |
| Overall p value | | | |
| Hard plus soft end point | | | |
| Log (anti-mhsp65) | | | |
| ≥90th centile anti-mhsp65 | | | |
| <9 | 1.06 (0.77 to 1.46) | 0.12 | 1.02 (0.59 to 1.80) | 0.92 | 0.36 | |
| ≥9–<17 | 0.76 (0.53 to 1.10) | 0.20 | 0.94 (0.29 to 3.00) | 0.96 | 0.80 | |
| ≥17–<31 | 0.76 (0.53 to 1.10) | 0.20 | 0.94 (0.29 to 3.00) | 0.96 | 0.80 | |
| ≥31 | 0.94 (0.67 to 1.30) | 0.69 | 0.94 (0.67 to 1.32) | 0.74 | |
were found significantly more often among patients than among controls (13.2% v 6.6%, p = 0.008). Median titres of anti-huhsp60 were similar in patients and controls.

The fourth study 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 with increased CRP and increased C pneumoniae IgA antibody titre conferred an odds ratio of 5.0 (95% CI 1.8 to 14.2). This 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 in the proportion of patients who had had a previous MI. In our group only 19.9% had a previous MI compared with 66.5% of patients in their study. Previous MI has been suggested to result in an important decrease in serum concentration of anti-huhsp60 antibodies. It seems likely, therefore, that titres of anti-huhsp60 and the anti-huhsp60-huhsp60 reaction may have different 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. Our study is the first to confirm this in the unstable angina population.

We confirmed the findings of Veres et al 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, mhsp65 is perhaps surprising because, like all hsp families, hsp60/65 is important decrease in serum concentration of anti-huhsp60 antibodies. It seems likely, therefore, that titres of anti-huhsp60 and the anti-huhsp60-huhsp60 reaction may have different 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. Our study is the first to confirm this in the unstable angina population.

ACKNOWLEDGEMENTS
This research study was funded by a British Heart Foundation Junior Research Fellowship (LEV) and project grant (DHB). Thanks to C McNeil for technical help. Recombinant mhsp65 was a gift from Dr H A van Embden, National Institute of Public Health and Environmental Protection, Biltoven, the Netherlands.

Authors’ affiliations
D H Birnie, LE Vickers, SM Cobbe, Department of Medical Cardiology, Royal Infirmary, Glasgow, UK
W S Hillis, Department of Medicine and Therapeutics, University of Glasgow, Glasgow, UK
J Norrie, Centre for Randomised Healthcare Trials (ChART), Health Services Research Unit, Aberdeen University, Aberdeen, UK

This research study was funded by a British Heart Foundation Junior Research Fellowship (LEV) and project grant. There are no conflicts of interest to declare.

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

REFERENCES
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 STEMI 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 thrombus suction. 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 thrombosis 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 × 5 × 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.

J-Y Wang  
L-C Lin  
anneiejou@ms28.hinet.net