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ORIGINAL ARTICLE

Plasma antibody titres to heat shock proteins-60, -65 and -70: Their relationship to coronary risk factors in dyslipidaemic patients and healthy individuals

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Abstract

Objective. To investigate the factors that may affect antibody titres to heat shock proteins (Hsp)-60, -65 and -70, and serum C-reactive protein (CRP) concentrations in patients with dyslipidaemia and other features of the metabolic syndrome as defined by ATPIII criteria. ***Material and methods.*** The study comprised 237 dyslipidaemia patients and 135 healthy individuals recruited from amongst university and hospital employees. ***Results.*** Compared to the healthy individuals, the dyslipidaemic patients had higher antibody titres to Hsp-60 ($p<0.01$), Hsp-65 ($p<0.001$) and Hsp-70 ($p<0.05$), and higher serum CRP concentrations ($p<0.001$). The best-fitting multifactorial models revealed that known coronary risk factors explained little of the variation in Hsp antibody titres: 3 % for Hsp-60, 1 % for Hsp-65 and 4 % for Hsp-70 amongst the dyslipidaemic subjects. The corresponding values for the subgroup with the metabolic syndrome were 8 %, 3 % and 1 %, respectively. In contrast, the best-fitting model explained 13.5 % of the variation in serum CRP concentrations among the dyslipidaemic patients, obesity being a major determinant; and 14 % in the subgroup with metabolic syndrome. ***Conclusions.*** The higher antibody titres to Hsp-60, -65, and -70 in the dyslipidaemic patients may be related to a heightened state of immunoactivation associated with atherosclerosis in this group. Our data indicate that antibody titres to these Hsps are not associated with the classical coronary risk factors, although serum high sensitivity (hs)CRP concentrations were significantly related to obesity.

Key Words: *CRP, BMI, IgG, metabolic syndrome, obesity*

Introduction

The inflammatory nature of atherosclerosis has been recognized for several decades [1]; serum inflammatory markers are predictive of atherosclerosis-related events [2,3] and this

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may be due to the impact of inflammation on plaque stability. The local inflammatory response appears to be associated with an autoimmune response to altered plaque components; these include modified forms of low-density lipoprotein (LDL) [4] and the heat shock proteins (Hsps). The latter are a family of approximately two dozen molecules expressed by cells in response to environmental stress, such as exposure to high temperature, free radicals, sheer stress and toxins, including oxidized LDL [5]. They are involved in the renaturation of damaged proteins, allowing the proteins to refold into their native conformation. It has been proposed that, because the structure of Hsps is highly conserved across species, the immune response mounted against bacterial Hsps may result in an autoimmune response [6]. This response has the potential to cause complement-mediated endothelial injury [7], and hence to accelerate atherogenesis. Antibodies to Hsp-60, -65 and -70 have previously been reported to be associated with some individual coronary risk factors [8], overall risk of vascular disease [9], its severity [10] and vascular end-points in patients with established disease [11]. The expression of Hsps is up-regulated in atherosclerotic plaque [12], and immunization of experimental animal models with human Hsp-60, or BCG vaccine (which contains high levels of Hsp-65) enhances the atherogenic process [13]. In the present study we have investigated the factors that could contribute to anti-Hsp antibody titres, including the inflammatory marker C-reactive protein (CRP), in healthy individuals, and in patients with dyslipidaemia and features of the metabolic syndrome as defined by ATPIII criteria [14].

Material and methods

Subjects

A total of 237 patients were recruited sequentially from the Lipid Clinics at the Royal Surrey County Hospital. No specific exclusion criteria were applied, apart from their being able to give informed consent; hence the population was representative of a Lipid Clinic in the UK. One hundred and eighty-nine healthy individuals were recruited from among employees at the University of Surrey and the Royal Surrey County Hospital, Guildford, UK. None of these subjects had a history of coronary heart disease (CHD), diabetes mellitus or hypertension. Of the control subjects, 54 were excluded from the subsequent analysis, as they were obese ($n=33$), or had the metabolic syndrome ($n=9$), or were taking medication ($n=12$).

Within the dyslipidaemic group there was a high frequency of obesity (body mass index (BMI) >30 kg/m^2), type 2 diabetes (fasting blood glucose >7 mmol/L), established CHD (including unstable angina, myocardial infarction, coronary artery bypass graft), systolic blood pressure $\geqslant 160$ mmHg or diastolic blood pressure $\geqslant 85$ mmHg, hypertriglyceridaemia (serum triglycerides >1.8 mmol/L), hypercholesterolaemia (serum total cholesterol >5.2 mmol/L) and the metabolic syndrome as defined by ATP III criteria (Table I).

Each subject gave informed written consent to participate in the study, which had previously been given approval by the South-West Surrey Research Ethics Committee and Surrey University's Advisory Committee. The characteristics of the patients and healthy subjects are presented in Table I.

Anthropometric and other measurements

All subjects were measured for height (in centimetres) and weighed in kilograms using a stand-on Bio Impedance Analyser (BIA) (Tanita-305 body fat analyser; Tanita Corp.,

Table I. Clinical characteristics of dyslipidaemic patients and healthy individuals.

	n (%)
Patients	237 (100)
Obese (body mass index >30 kg/m^2)	82 (35)
Diabetes type II (fasting blood glucose >7 mmol/L)	42 (18)
Established coronary heart disease	55 (23)
Unstable angina	9 (4)
Myocardial infarction (MI)	15 (6)
Coronary artery bypass graft (CABG)	10 (4)
Angioplasty	13 (5.5)
Angioplasty or CABG after MI	8 (3)
Hypertension	186 (79)
High BP (SBP 160 mmHg or DBP $\geqslant$100 mmHg)*	76 (32)
Moderate BP (130$\leq$SBP$<$160 mmHg or 85$\leq$DBP$<$100 mmHg)	110 (46)
Hypertriglyceridaemia (serum triglycerides >1.8 mmol/L)	176 (74)
Hypercholesterolaemia (serum total cholesterol >5.2 mmol/L)	216 (91)
Calculated 10-years coronary risk >30 % (calculated using the PROCAM algorithm)	42 (18)
Calculated 10-years coronary risk between 20 and 30 %	54 (23)
Metabolic syndrome (as defined using NCEP-ATP III criteria)	142 (60)
Controls	189 (100)
Healthy individuals	135 (70)
Excluded subjects	57 (30)
Obese	33 (17)
Metabolic syndrome	9 (5)
Taking medication	12 (6)

*Systolic and diastolic blood pressure; SBP and DBP.

Tokyo, Japan). The latter was also used to measure percentage of body fat. BMI was calculated using the formula: BMI = weight (kg)/height (m^2).

Waist and hip measurements were taken to the nearest millimetre as previously described [15]. Blood pressure measurements were made using an automated device (DINAMAP compact monitor, model TS; Critikon, Tampa, Fla., USA, FA 33634).

Blood sampling

Blood samples were collected between 0830 h and 1030 h after a 12-h fast. Following venepuncture of an antecubital vein, blood samples were collected into Vacutainer tubes (Becton-Dickinson, Cowley, Oxford, UK), and centrifuged at 10,000*g* for 15 min at 4°C. After separation, aliquots of serum were frozen at −80°C until the day of analysis.

Biochemical analysis

All chemicals were obtained from Sigma (Sigma Chemical Co., Poole, Dorset, UK) unless stated otherwise.

Lipid profiles and blood glucose

A full, fasted lipid profile comprising total cholesterol, triglycerides and high-density lipoprotein (HDL) cholesterol was determined for each patient. LDL cholesterol was calculated using the Friedewald equation [16], except for patients with serum triglycerides >4.0 mmol/L. LDL values in patients with triglycerides >4 mmol/L were omitted from

the data analysis. Lipid and blood glucose measurements were estimated using routine enzymatic methods on a Bayer Advia 1650 analyser (Bayer, Newbury, UK).

High-sensitivity C-reactive protein (hs-CRP)

High-sensitivity serum CRP concentrations were determined by PEG enhanced immunoturbidimetry on a Bayer Advia 1650 autoanalyser.

Hsp antibody titres

Serum Hsp antibody titres were measured using in-house ELISAs, using a modification of the method previously described by Xu et al. [17]. In brief, a 96-well microtitre plate (Nunc Immunoplate Maxisorp; Life Technologies, UK) was coated with human recombinant Hsp-60, -65 or -70 by adding 10 ng recombinant Hsp in phosphate buffered saline (PBS) per well and incubating overnight at 4°C. The plates were washed with PBS, blocked with Superblock (Pierce & Wariner, Chester, Cheshire, UK) and washed three times with PBS containing 0.05 % vol/vol Tween-20. Serum samples were diluted 1:15 with PBS containing 0.1 % Tween-20 and 1 % bovine serum albumin (PBT) and a 100 μL/well in quadruplicate incubated for 30 min at 37°C. After washing, bound anti-Hsp antibodies were detected by adding peroxidase conjugated goat anti-human IgG, diluted at 1:100 with PBT (Sigma-Aldrich Inc., USA). After washing with PBS/Tween-20, o-phenylenediamine in citrate/phosphate/hydrogen, peroxide was added and incubated for 5 min. The reaction was terminated by the addition of 3 M hydrochloric acid. The absorbance was read at 492 nm using a plate reader with Genesis 2 Software (Life Sciences, Basingstoke, Hampshire, UK). The within-batch and between-batch CVs were <5 % and <8 %, respectively.

Statistical analysis

Minitab software (Release 13; Minitab Inc., Pa., USA) was used for the statistical analysis. Quantitative data were assessed for normality using the Kolmogorov-Smirnov tests. Comparisons were made between the healthy subjects and dyslipidaemic patients and between different subcategories of the dyslipidaemic patients (obese versus non-obese; metabolic syndrome versus non-metabolic syndrome, etc.).

Data that were normally distributed were analysed using one-way analysis of variance (ANOVA). Data found to be non-normally distributed were analysed using the non-parametric Kruskal-Wallis test. A two-sided p-value of <0.05 was considered statistically significant.

Values were expressed as means and standard error of the mean (SEM) or, in the case of non-normally distributed data, as median and interquartile range. Categorical data (such as smoking habit) were analysed using Fisher's exact or χ^2 tests.

Multiple linear regression analysis was used to investigate the relationship between the anti-Hsp titres and the hs-CRP concentration and possible coronary risk factors and individual components of the metabolic syndrome. Forward and backward stepwise regression was used in which the Hsp titres and hs-CRP concentrations were entered, in turn, as the dependent variable. The independent variables included: age, gender, diabetes mellitus, the metabolic syndrome, obesity, hypertriglyceridaemia, smoking, hypertension, history of CHD, accumulation features of the metabolic syndrome, and drug therapy for dyslipidaemic patients, and systolic and diastolic blood pressure, waist measurements,

BMI, waist: hip ratio, fasting blood glucose, triglycerides and HDL levels for the metabolic syndrome group.

The stepwise analysis used the Minitab default value of $p=0.15$ as the criterion for inclusion or exclusion of a variable from the model. The results of the multiple linear regression analyses were assessed by assessing how well the models accorded with the data, the proportion of the variability in the dependent variable explained (R^2) the size and statistical significance of any terms included in the model, the pattern of residuals and the impact of possible outliers and points with high leverage.

Results

Characteristic of patients and healthy individuals

There was a high frequency of obesity (35 %), type 2 diabetes (18 %), hypertension (79 %) and positive smoking habit (18 %) in the patient group (Table I). These findings are typical of a Lipid Clinic population. Serum fasting triglycerides, blood glucose levels and total and LDL cholesterol were significantly higher than those for controls, as would be expected for patients attending such a clinic. Anthropometric indices including waist circumference, waist:hip ratio, BMI and percentage body fat were also higher in the patients compared with the healthy individuals (Table II). Current smoking habit did not differ between patients and healthy subjects; however, a greater proportion of the patients were former smokers (Table II). The patients were also on average nearly 6.5 years older than healthy

Table II. Comparison of clinical and biochemical characteristics of dyslipidaemic patients and healthy individuals.

Group	Patients	Healthy individuals	*p*-value	Gender differences*
Number	237	135	–	–
Mean age (years)	55.2±0.86	48.9±1.26	<0.001	–
Male:Female ratio	142/95 (60 %)	67/68 (50 %)	0.14	–
Smoking habit				
Current no. (%)	43 (18)	23 (17)	0.90	0.15
Former no. (%)	84 (35)	25 (19)	<0.001	0.008
Never no (%)	110 (46)	87 (64)	<0.001	0.002
Body mass index (kg/m^2)	29.1±0.33	24.3±0.25	<0.001	0.80
Body fat (%)	32.5±0.53	26.6±0.63	<0.001	<0.001
Waist circumference (cm)	98.1±0.83	85.7±0.90	<0.001	<0.001
Waist:hip ratio	0.93±0.01	0.86±0.01	<0.001	0.001
Systolic blood pressure (mmHg)	146.7±1.32	125.2±1.38	<0.001	0.58
Diastolic blood pressure (mmHg)	82.1±0.77	74.7±0.79	<0.001	0.007
Total cholesterol (mmol/L)	7.38±0.12	5.41±0.09	<0.001	0.14
HDL cholesterol (mmol/L)	1.27±0.03	1.70±0.04	<0.001	<0.001
Triglycerides (mmol/L)	2.61 (1.71–4.5)	1.06 (0.86–1.39)	<0.001	0.049
Fasting blood glucose (mmol/L)	5.92±0.10	4.99±0.04	<0.001	0.91

Values are expressed as means±SEM, or median and interquartile range. Categorical data were compared using Fisher's exact test. Between-group comparisons were assessed using the Kruskal-Wallis test for non-normal distribution data (serum triglycerides) and by one-way ANOVA for normally distributed data.
*Comparison of parameters in male and female patients shown in this column.

subjects ($p<0.001$). The difference in the proportion of males in the groups (60% of patients compared with 50 % of healthy subjects) did not differ significantly.

Correlation between CHD risk factors and anti-Hsp titres

Univariate analysis between serum Hsp-60, -65 and -70 antibody titres and individual coronary risk factors, showed that serum Hsp-70 antibody titres were correlated with serum triglycerides in the healthy subjects ($p<0.05$, Table III). In the patient group, antibody titres to Hsp-60 were associated with fasting blood glucose ($p<0.05$), and Hsp-65 antibody titres were associated with serum HDL, waist:hip ratio and systolic and diastolic blood pressure ($p<0.05$, Table IV).

Anti-Hsp antibody titres in dyslipidaemic patients and healthy subjects

Overall, dyslipidaemic patients had significantly higher antibody titres to Hsp-60 ($p<0.01$), Hsp-65 ($p<0.001$) and Hsp-70 ($p<0.05$) compared with those of healthy

Table III. Correlations (r) between Hsp antibody titres and serum CRP concentrations with CHD risk factors in healthy individuals.

	Anti-Hsp-60	Anti-Hsp-65	Anti-Hsp-70	CRP
Age	0.11	0.16	−0.16	0.18*
Fasting blood glucose	0.10	−0.00	−0.02	0.06
BMI	−0.04	−0.04	−0.07	0.42***
Waist:hip ratio	0.10	−0.01	−0.14	0.17*
HDL cholesterol	0.02	0.17	−0.03	−0.07
Triglycerides	−0.11	−0.14	−0.19*	0.27***
Systolic blood pressure	0.11	0.10	−0.03	0.25**
Diastolic blood pressure	0.10	0.09	0.02	0.19**
Hs-CRP	0.00	0.06	−0.06	–

Abbreviations: Hsp=heat shock protein; CRP=C-reactive protein; CHD=coronary heart disease; BMI=body mass index; HDL=high-density lipoprotein; hs-CRP=high-sensitivity CRP.
*$p<0.05$; **$p<0.01$; ***$p<0.001$.
Hsp antibody titres and serum CRP concentrations were log-transformed before using the Pearson correlation.

Table IV. Correlations (r) between Hsp antibody titres and serum CRP concentrations with CHD risk factors in dyslipidaemic patients.

	Anti-Hsp-60	Anti-Hsp-65	Anti-Hsp-70	CRP
Age	0.07	0.06	0.04	0.11
Fasting blood glucose	0.13*	−0.02	−0.03	0.10
BMI	0.01	0.09	0.11	0.42***
Waist:hip ratio	0.10	0.13*	0.08	0.28***
HDL cholesterol	−0.08	−0.14*	−0.05	−0.06
Triglycerides	−0.01	−0.00	0.02	0.26***
Systolic blood pressure	0.05	0.16*	0.02	0.02
Diastolic blood pressure	0.09	0.16*	0.05	0.06
Hs-CRP	0.09	−0.01	0.09	–

Abbreviations: CRP=C-reactive protein; CHD=coronary heart disease; BMI=body mass index; HDL=high-density lipoprotein; Hsp=heat shock protein; hs-CRP=high-sensitivity CRP.
*$p<0.05$; ***$p<0.001$.
Hsp antibody titres and serum CRP concentrations were log-transformed before using the Pearson correlation.

subjects (Table V). Male dyslipidaemic patients had significantly higher serum antibody titres to Hsp-60 ($p<0.01$), Hsp-65 ($p<0.01$) and Hsp-70 ($p<0.05$) compared with those of healthy subjects (Table V). Female dyslipidaemic patients had significantly higher anti-Hsp-65 titres ($p<0.001$) than healthy females, whereas their antibody titres to Hsp-60, and Hsp-70 did not differ significantly (Table V).

In general, antibody titres to Hsps remained significantly higher in patients compared with healthy subjects for subcategories of dyslipidaemic patients, even for those subcategories without the specific coronary risk factor (Table VI).

Strong correlations were found between Hsp antibody titres within each respective subject group (Hsp-60 versus Hsp-65, Hsp-60 versus Hsp-70, Hsp-65 versus Hsp-70) ($p<0.001$ in each case).

Hsps antibody titres did not differ significantly between male and female patients or healthy subjects. Nor did titres differ significantly between subjects who were smokers and those who were non-smokers.

Correlation between CHD risk factors and serum hs-CRP

Serum hs-CRP concentrations were positively associated with BMI, and serum triglycerides in the patients and healthy individuals ($p<0.001$, Tables III and IV). In the healthy subjects, serum hs-CRP concentrations were also positively associated with age ($p<0.05$) and systolic and diastolic blood pressure ($p<0.05$, Table III).

Table V. Comparison of Hsp antibody titres (absorbance units) between dyslipidaemic patients and healthy individuals: effects of gender.

	Patients	Healthy individuals
Males		
No. of subjects	142	67
Anti-Hsp-60	0.26 (0.19–0.38)**	0.21 (0.17–0.32)
Anti-Hsp-65	0.45 (0.27–0.78)**	0.33 (0.22–0.54)
Anti-Hsp-70	0.23 (0.17–0.30)*	0.19 (0.13–0.26)
Hs-CRP (mg/L)	1.08 (0.37–3.19)***	0.41 (0.16–0.93)†
Females		
No. of subjects	95	68
Anti-Hsp-60	0.27 (0.16–0.36)	0.23 (0.15–0.28)
Anti-Hsp-65	0.45 (0.29–0.81)***	0.29 (0.21–0.49)
Anti-Hsp-70	0.20 (0.16–0.30)	0.19 (0.14–0.29)
Hs-CRP (mg/L)	1.54 (0.50–4.47)**	0.94 (0.20–2.14)
Males & Females combined		
No. of subjects	237	135
Anti-Hsp-60	0.27 (0.18–0.37)**	0.22 (0.16–0.30)
Anti-Hsp-65	0.45 (0.28–0.79)***	0.31 (0.22–0.50)
Anti-Hsp-70	0.22 (0.17-0.30)*	0.19 (0.13–0.27)
Hs-CRP (mg/L)	1.25 (0.42–3.26)***	0.58 (0.17–1.42)

Abbreviations: Hsp=heat shock protein; CRP=C-reactive protein; hs-CRP=high-sensitivity CRP.
Values are expressed as medians and interquartile range. Between-group comparisons were assessed with the Kruskal-Wallis test as they are non-normal distribution data.
$^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ compared to healthy subjects; $^{\dagger}p<0.05$ compared to healthy females.

Table VI. Serum heat shock protein antibody titres in different subgroups of dyslipidaemic patients segmented according to the possession of specific coronary risk factors, and in healthy subjects.

	n	Anti-Hsp-60	Anti-Hsp-65	Anti-Hsp-70
Group				
Established CHD				
CHD+	55	0.27 (0.19–0.35)**	0.43 (0.29–0.87)***	0.24 (0.18–0.36)**†
CHD−	182	0.26 (0.18–0.38)	0.46 (0.26–0.77)	0.21 (0.16–0.30)
Metabolic syndrome				
MS+	142	0.27 (0.19–0.38)**	0.47 (0.29–0.80)***	0.23 (0.17–0.31)*
MS−	95	0.27 (0.16–0.36)	0.39 (0.25–0.77)	0.20 (0.16–0.28)
Diabetes mellitus				
DM+	42	0.23 (0.18–0.36)**	0.44 (0.29–0.82)***	0.26 (0.18–0.34)**†
DM−	195	0.27 (0.18–0.37)	0.45 (0.27–0.79)	0.22 (0.16–0.28)
Obesity				
Obese+	82	0.27 (0.19–0.38)**	0.46 (0.30–0.83)**	0.24 (0.17–0.30)*
Obese−	155	0.27 (0.17–0.37)	0.44 (0.26–0.78)	0.21 (0.16–0.29)
Hypertriglyceridaemia				
High TG	176	0.27 (0.19–0.39)***†	0.46 (0.29–0.77)***	0.23 (0.17–0.30)*
Normal TG	61	0.25 (0.16–0.33)	0.43 (0.25–0.87)	0.19 (0.16–0.28)
Blood pressure				
High BP	76	0.27 (0.18–0.38)*	0.47 (0.30–0.92)***	0.23 (0.17–0.30)
Moderate BP	111	0.27 (0.19–0.39)	0.43 (0.28–0.69)	0.20 (0.160.32)
Normal BP	50	0.25 (0.17–0.35)	0.41 (0.25–0.78)	0.22 (0.17–0.28)
Calculated 10-years coronary risk				
High >30 %	42	0.28 (0.20–0.51)**	0.50 (0.35–0.89)***	0.23 (0.17–0.33)
Moderate 20–30 %	54	0.25 (0.17–0.35)	0.40 (0.26–0.75)	0.21 (0.17–0.28)
Low <20 %	141	0.26 (0.17–0.37)	0.45 (0.28–0.79)	0.22 (0.16–0.30)
Healthy subjects	135	0.22 (0.16–0.30)	0.31 (0.22–0.50)	0.19 (0.13–0.27)

Abbreviations: Hsp=heat shock protein; CHD=coronary heart disease.
Values are expressed as median and interquartile range. Between-group comparisons were assessed by Kruskal-Wallis tests as they were non-normally distributed data. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ for comparison between subcategories of the dyslipidaemic patients and healthy subjects, and $^{\dagger}p<0.05$ comparison between subcategories of the dyslipidaemic patients.

Serum hs-CRP concentrations in dyslipidaemic patients and healthy individuals

Serum hs-CRP concentrations were significantly higher in patients compared with healthy subjects, for males ($p<0.001$), females ($p<0.01$) and the combined group ($p<0.001$) (Table V). Among the healthy subjects, males had significantly lower serum hs-CRP concentrations than those of female subjects ($p<0.05$); there was no significant gender difference in the dyslipidaemic group, nor did serum hs-CRP concentrations differ significantly between smokers and non-smokers for the group as a whole. However, among healthy subjects, female smokers had significantly higher serum hs-CRP concentrations than those of non-smokers ($p<0.05$).

Within the dyslipidaemic group, serum hs-CRP concentrations rose with accumulating features of the metabolic syndrome ($p<0.01$, Table VII), although this was not the case for any of the serum Hsp antibody titres (Table VII). Serum CRP concentrations also differed significantly between subcategories of dyslipidaemic subjects, classified according to the following features: the presence of CHD ($p<0.05$), the metabolic syndrome ($p<0.001$), diabetes mellitus ($p<0.05$), obesity ($p<0.001$) and calculated 10-years coronary risk scores ($p<0.05$, Table VIII).

Table VII. Serum hsCRP and heat shock protein antibody titres in dyslipidaemic patients with different features of the metabolic syndrome.

No. of features	0	1	2	3	4	5
No. of subjects (*n*)	10	28	61	62	56	20
CRP (mg/L)						
Median and interquartile	0.51	0.79	0.79	1.86	1.60	2.37**
Range	(0.21–2.42)	(0.21–2.82)	(0.36—2.03)	(0.51–5.14)	(0.64–4.78)	(0.85–4.35)
Antibody titre (absorbance)						
Median and interquartile						
Range						
Hsp-60	0.30	0.23	0.27	0.28	0.28	0.24
	(0.16–0.34)	(0.14–0.29)	(0.17–0.39)	(0.19–0.42)	(0.19–0.36)	(0.17–0.37)
Hsp-65	0.32	0.42	0.46	0.46	0.45	0.53
	(0.15–0.40)	(0.26–0.87)	(0.25–0.73)	(0.29–0.86)	(0.32–0.76)	(0.30–0.84)
Hsp-70	0.21	0.18	0.22	0.23	0.24	0.26
	(0.14–0.28)	(0.15–0.24)	(0.17–0.33)	(0.16–0.30)	(0.17–0.30)	(0.18–0.36)

Abbreviations: Hsp=heat shock protein; CRP=C-reactive protein.
Between-group comparisons were assessed with the Kruskal-Wallis test as they are non-normal distribution data.
** $p<0.01$.
One-way ANOVA analysis of log-transformed data showed that patients with three or more features of the metabolic syndrome had significantly higher serum CRP levels compared with patients with two or fewer features of the metabolic syndrome.

Analysis of covariance revealed that when BMI was entered as a covariate, the difference between the serum CRP of patients and healthy subjects was no longer significant.

Multiple regression analysis

In general, little of the variation in the anti-Hsp titres of the 237 dyslipidaemic patients could be explained by the best-fitting models derived from stepwise multiple linear regression: 3 % for Hsp-60, 1 % for Hsp-65 and 4 % for Hsp-70. In the 142 individuals with the metabolic syndrome the corresponding values were 8 %, 3 % and 1 % for the three anti-Hsp titres respectively. In the 135 healthy subjects, again the best-fitting models derived from stepwise multiple linear regressions explain little of the variation in Hsp antibody titres: 4 % for Hsp-60, 3 % for Hsp-65, and 4 % for Hsp-70. This was in agreement with the results obtained from the univariate analyses.

In the case of CRP, the best model explained 13.5 % of the variation in the dyslipidaemic patients (obesity was the major determinant), 14 % in the group with the metabolic syndrome and 10 % in the healthy subjects, the major determinant being BMI. Again, this was consistent with the results obtained from the univariate analysis (Tables III and IV). Adding extra terms to the model had little effect on explaining the variability in CRP values in the patients, as estimated by the adjusted R^2 values.

Discussion

The relationship between serum inflammatory markers such as CRP and CHD has been shown in cohort studies [3] and has been supported by meta-analysis data [2]. More recently, levels of serum CRP have been reported to be related to individual coronary risk

Table VIII. Serum hs-CRP concentrations in different subgroups of dyslipidaemic patients segmented according to the possession of specific coronary risk factors, and in healthy subjects.

	n	CRP
Group		
Established CHD		
CHD+	55	1.80 (0.43–6.09)$^{\dagger\dagger\dagger *}$
CHD−	182	1.17 (0.42–2.96)†††
Metabolic syndrome		
MS+	142	1.70 (0.60–4.57)$^{\dagger\dagger\dagger ***}$
MS−	95	0.79 (0.29–2.27)†††
Diabetes mellitus		
DM+	42	2.06 (0.62–5.79)$^{\dagger\dagger\dagger *}$
DM−	195	1.10 (0.41–3.04)†††
Obesity		
Obese+	82	2.82 (1.03–5.81)$^{\dagger\dagger\dagger ***}$
Obese−	155	0.85 (0.33–2.04)†††
Hypertriglyceridaemia		
High TG	176	1.54 (0.49–3.23)†††
Normal TG	61	0.74 (0.32–3.99)†††
Blood pressure		
High BP	76	1.61 (0.49–3.47)†††
Moderate BP	111	1.25 (0.39–3.19)†††
Normal BP	50	1.22 (0.46–3.55)†††
Calculated 10-years coronary risk		
High >30 %	42	2.71 (0.49–6.10)$^{\dagger\dagger\dagger *}$
Moderate 20–30 %	54	1.14 (0.45–4.70)†††
Low <20 %	141	0.98 (0.40–2.66)†††
Healthy subjects	135	0.58 (0.17–1.42)

Abbreviations: CHD=coronary heart disease; CRP=C-reactive protein.
Values are expressed as median and interquartile range. Between-group comparisons were assessed by Kruskal-Wallis test as they were non-normally distributed data.
$^{\dagger\dagger\dagger}p<0.001$ for comparison with healthy individuals; $^{*}p<0.05$; $^{***}p<0.001$ for comparison between subcategories of dyslipidaemic patients.

factors including features of the metabolic syndrome [18]. The reported association between antibody titres to the Hsps and atherosclerosis has been less consistent [19,20].

Serum CRP concentrations and CHD risk factors

We found a strong relationship between CRP and indices of adiposity in subjects with dyslipidaemia, as previously reported for other groups of subjects [21]. However, this association does not necessarily indicate causality. We, like Tamakoshi and colleagues [22] and Ridker et al. [23], found that CRP concentrations rose with increasing number of features of the metabolic syndrome, and the relative values were similar in our Caucasian population to those reported in their populations. These investigators have also reported a positive association between fasting glucose, triglycerides and systolic blood pressure and a negative association with HDL cholesterol [22]. We found no relationship between fasting blood glucose levels and CRP as has been reported by others [24].

Hsp antibody titres, CHD risk and the metabolic syndrome

Pockley et al. [25] have reported that Hsp-70 and Hsp-65 antibody titres were elevated in subjects with hypertension, whereas serum Hsp-60 and Hsp-70 antigen concentrations and

Hsp60 antibody titres were similar for hypertensive subjects and normotensive controls. Antibody titres to Hsp-60, -65, and -70 have also been reported to be positively related to risk of vascular disease and cardiovascular end-points. Although high Hsp-60 IgA antibody titres were predictive of coronary risk, the effects were reported as modest in the absence of other classical risk factors [26]. High serum levels of Hsp-70 antigen have been reported to be associated with low coronary artery disease (CAD) risk, possibly because of its multiple cytoprotective effects [27]. Kocsis et al. have recently reported that anti-Hsp-70 titres are not elevated in subjects with severe coronary atherosclerosis, whereas titres to Hsp-60 and -65 were significantly raised in these patients, there being no association between antibody titres against Hsp-70 with either Hsp-60, or -65 [28]. Our findings were similar to those reported by Kocsis et al. [28] with significantly higher titres to Hsp-60 and -65 in patients compared with those in healthy individuals.

The associations between individual coronary risk factors and accumulating features of the metabolic syndrome with antibody titres to all three Hsps were weak for both the healthy and dyslipidaemic subjects. This suggests that that there are likely to be determinants of serum antibody titres to Hsps other than traditional coronary risk factors. The reported associations between Hsp antibody titres and cardiovascular risk may be related to some of these other determinants. For example, the relationship between antibody titres to Hsp and infection has been reported elsewhere [29]. We have also recently reported that dietary factors, including antioxidant vitamins and saturated fat, may modulate Hsp antibody titres [30]. These factors could not be included in our model and may to some degree explain why our best-fitting models explained a relatively small amount of the variation in the antibody titres to the Hsps.

Conclusions

The high antibody titres to Hsp-60, -65 and -70 in dyslipidaemic patients may be related to a heightened state of immunoactivation associated with atherosclerosis in this group, and the strong correlations between antibody titres to Hsp-60, -65 and -70 ($p < 0.001$) supports this possibility. However the lack of significant correlations between serum hs-CRP concentrations and any of the Hsp antibody titres does not favour this hypothesis. Our data also suggest that antibody titres to Hsp-60, -65 and -70 are independent of classical coronary risk factors. By contrast, serum hs-CRP concentrations were significantly related to obesity or to BMI.

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