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Association between antibodies to heat shock protein 65 and coronary atherosclerosis

Possible mechanism of action of *Helicobacter pylori* and other bacterial infections in increasing cardiovascular risk

D. H. Birnie*, E. R. Holme†, I. C. McKay†, S. Hood*, K. E. L. McColl* and W. S. Hillis*

**Department of Medicine & Therapeutics and †Immunology, University of Glasgow, Western Infirmary, Glasgow, U.K.*

Introduction There is growing evidence that the immune response is involved in atherosclerosis. Antibodies to heat shock protein 60/65 have been shown to be a risk factor for carotid atherosclerosis and been proposed as a diagnostic marker of atherosclerosis. In addition, it has been suggested that the immune response to heat shock protein 60/65 may be a link between exposure to microorganisms and increased cardiovascular risk.

Aims (1) To investigate the association between anti-shock protein 65 titre and coronary atherosclerosis; (2) To assess whether anti-mhsp65 titre is a useful diagnostic marker of atherosclerosis; (3) To examine the influence of *Helicobacter pylori* infection on anti-heat shock protein 65 titre.

Methods and Results In the first study we measured anti-heat shock protein 65 titres in 136 consecutive male subjects admitted for routine coronary angiography. Anti-heat shock protein 65 titres correlated with both the severity and extent of coronary atherosclerosis and the relationship remains statistically significant for the presence of atherosclerosis ($P = 0{\cdot}012$) after adjustment for possible confounding influences. However the association had insufficient sensitivity to be a useful clinical test. In the second study we recruited 100 patients with confirmed active *H. pylori* infection and double blindly randomized them to eradication therapy or placebo. Successful eradication of *H. pylori* led to a significant fall in anti-heat shock protein 65 titres (from a mean of $256{\cdot}4\ \mathrm{AU} \cdot \mathrm{ml}^{-1}$ to $137{\cdot}5\ \mathrm{AU} \cdot \mathrm{ml}^{-1}$, $P = 0{\cdot}033$).

Conclusion These results raise the possibility that exposure to *H. pylori* and other micro-organisms lead to an increased risk of clinically manifest coronary artery disease by an autoimmune process.
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Key Words: Heat shock protein 65, anti-mhsp65, coronary atherosclerosis, *Helicobacter pylori*, auto-immunity.

Introduction

Recent data has suggested that *Helicobacter pylori* is a risk factor for coronary heart disease[1–3]. However a larger study[4] found no residual relationship between *H. pylori* infection and coronary artery disease after correction for age and social class. This highlights the difficulty in investigating a residual relationship between *H. pylori* infection and coronary artery disease when each individually is strongly related to social class and age. The loss of significance after correcting for age and social class does not exclude a causal relationship between *H. pylori* and coronary artery disease as *H. pylori* infection could be a mechanism by which these factors increase the risk of coronary artery disease. Thus, it is important to test the hypothesis of a causal relationship in other ways and one of these is by investigating possible pathogenic processes. Previously it has been postulated that the association between *H. pylori* infection and ichaemic heart disease is due to systemic effects of *H. pylori* infection on fibrinogen concentration and total white blood cell count[3] but this hypothesis has been challenged[4]. An alternative hypothesis is that *H. pylori* infection leads to increased cardiovascular risk by an auto-immune process.

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Correspondence: Dr D. Birnie, Department of Medical Cardiology, Queen Elizabeth Building, Glasgow Royal Infirmary, Glasgow G31 2ER, U.K.

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Certainly there is growing evidence that the immune response is involved in the pathogenesis of atherosclerosis[5,6]. Much of the emerging data is about the immune response to heat shock proteins. Heat shock proteins are a group of about 24 proteins that show highly homologous sequences between different species from bacteria to man. Antibodies to mycobacterial heat shock protein 65 (anti-mhsp65) have been proposed as a diagnostic marker for the presence of atherosclerosis[7]. Xu and colleagues have postuled a role for mhsp65 and the very similar human hsp60/65 in a series of papers[8–12]. They injected a panel of antigens from atherosclerotic lesions and other sources and found that mhsp65 was unique in producing atherosclerosis in normocholesterolaemic rabbits[8]. Subsequently, increased expression of hsp60 was shown on endothelial cells, macrophages and smooth muscle cells of atherosclerotic lesions in rabbit and human arteries[9,10]. Hsp 60 was present on even the earliest lesions of the atherosclerotic process (fatty streaks) but not on normal arterial intima. Subjects with carotid atherosclerosis[11] and coronary artery disease[12] had significantly higher titres of anti-mhsp65 antibodies compared to controls. Increased hsp expression in and on cells is induced by stresses such as infection, high temperature, free radicals, mechanical stress and hypoxia. Heng and Heng[13] have shown increased hsp65 expression in ischaemic human arterial walls within 30 min of external ligation.

The anti-hsp60/65 antibody response in atherosclerosis may be a secondary phenomenon or, alternatively have a more causal role[11]. Evidence from a twin study in humans[14] and from an animal model[15] suggests that the induction of anti-hsp65 antibodies depends on environmental factors such as exposure to micro-organisms. *H. pylori* has been shown to surface express a 62 k dalton heat shock protein[16] which is 75% homologous to mhsp65 and human hsp60. Sharma *et al.*[17] demonstrated that this hsp62 antigen provokes an antibody response after *H. pylori* infection.

In this paper we report two studies. The first examines the relationship between anti-mhsp65 titres and coronary atherosclerosis, and the relationship of these antibodies and atherosclerosis with *H. pylori* antibodies and infection. The second examines the effect of *H. pylori* eradication therapy on anti-mhsp65 titre.

Methods

Subjects

In the first study we recruited 136 consecutive male subjects (age mean 55·3 years, range 31·2–79·2) admitted for elective cardiac catheterization. Subjects were excluded if they had any other disease associated with elevated anti-mhsp65 titre (reviewed in[18]), including diabetes mellitus (fasting glucose of greater than 7·8 mmol . l^{-1}), rheumatoid arthritis, SLE or had other evidence of active infection, inflammation or malignancy or had had previous coronary angioplasty or coronary artery bypass surgery. In the majority of cases, the angiography was indicated for the assessment of chest pain and a smaller group of patients were being investigated for valvular abnormalities. The subjects were recruited over a 7-month period and the population demographics are listed in Table 1. The study was approved by the hospital Ethical Committee and all patients gave informed consent. Patients filled in a short health questionnaire, including questions on past and concomitant health problems, history of hypertension, family history of atherosclerosis and smoking record. Smoking was treated as a categorical variable (smoker, non-smoker, ex-smoker) and a continuous variable (estimated lifetime number of packets consumed). Social class was assessed using deprivation categories derived from Carstairs scores of postcode sectors. These deprivation categories (DEPCATS 1–7) provide a measure of deprivation or affluence on the basis of a combination of selected 1991 Census variables standardized to their mean[19]. The variables used to create the scores were car ownership, male unemployment, head of household occupation and overcrowding.

Table 1 Description of cohort of 136 male subjects

Age mean (SD)	55·3 (10·4)
Body mass index (kg . m^{-2})	26·7 (3·7)
History of hypertension	30·9%
Family history of coronary atherosclerosis	47·5%
Never smoked	18·5%
Ex-smoker	61·4%
Current smoker	20·1%
Indication for angiography	
Chest pain	95·6%
Valvular abnormality	4·4%

In the second study we recruited 100 subjects (age mean 43·4 years, range 17–65, 40 males) all with active *H. pylori* infection confirmed by the urea breath test. The subjects were recruited over a 3-month period and the study was approved by the hospital Ethical Committee and all patients gave informed consent. The subjects were double blindly randomized to active treatment (n = 48) or placebo (n = 52). Active treatment consisted of omeprazole 20 mg b.d., metronidazole 400 mg t.i.d. and amoxycillin 500 mg t.i.d. for 14 days. The placebo group received 2 weeks of omeprazole 20 mg b.d. plus placebo antibiotics. The subjects returned one year after commencing therapy and had a further breath test. Subjects were excluded if the follow-up breath test result did not confirm successful eradication in the active treatment group or continuing infection in the placebo group. This left 33 in the treatment group and 41 in the placebo group.

Sample preparation

The patients fasted overnight and 40 ml of blood was removed the following morning prior to coronary

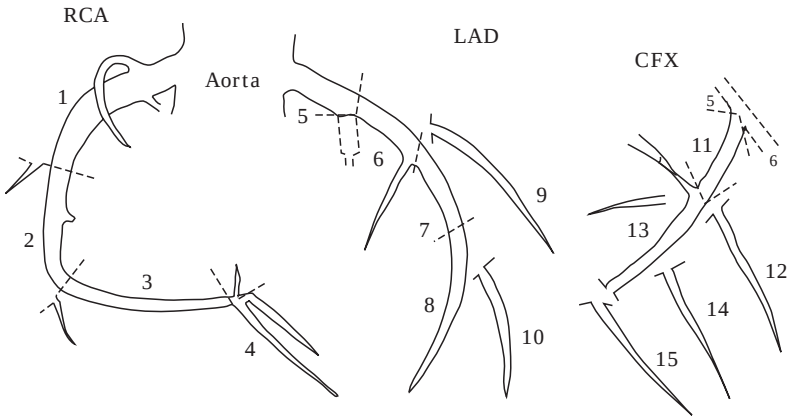

Figure 1 **The coronary circulation. Segments 1–3, 5–8, 11 and 13 were used to calculate the atherosclerotic severity score. Each segment was scored depending upon the severity of stenosis as follows: normal segment scoring 0, <50% stenosis — 1, 50–90% stenosis — 12·5; 91–99% stenosis — 20, 100% stenosis — 25 and the overall score was the sum of the 8 individual segments. All segments were used in the calculation of diffuseness score, the 8 proximal segments were first order and the 7 distal segments second order. The first order segments received a score of 1 if there was any evidence of atherosclerosis, and the second order segments scored 0·5. The overall diffuseness score was the sum of the 15 individual segment scores. Right Coronary Artery (RCA), Left Anterior Descending (LAD), circumflex (CFX).**

angiography or breath testing. Samples were centrifuged within 2 h of collection and the serum and plasma stored at −20 °C for subsequent assay.

Assays

The IgG anti-mhsp65 titres were measured by ELISA, by a modification of the method of Xu *et al.*[11] and as previously described by us[14]. IgG antibodies specific to *H. pylori* were quantified by using the BIO-Rad (California, U.S.A.) G.A.P. IgG test kit. This is an ELISA system using plates coated with *H. pylori* antigens. IgG-specific *H. pylori* antibodies are quantified in units/ml ($U \cdot ml^{-1}$) and a cut-off titre of 12 $U \cdot ml^{-1}$ has been shown to be 94·9% sensitive and 91·3% specific for culture proven *H. pylori* gastric infection[20]. Subjects in the first study also had cholesterol and subfractions, triglycerides and C-reactive protein assayed by standard techniques. Also, lipoprotein(a) and fibrinogen were assayed by standard immunoprecipitan analysis with Incstar calibration/control sets (Incstar, Wokingham, England, Catalogue nos 86116 and 86114, respectively) for calibration and using a Roche Cobas Bio Centrifugal Analyser. The samples were all assayed without prior knowledge of the coronary angiography findings.

Coronary angiography

This was performed using standard techniques and recorded in multiple projections for left and right coronary arteries. Scoring of the angiograms was performed by a single independent observer (W.S.H.). A random 10% were analysed a second time by the same observer blind to the first results to enable calculation of intra-observer variability. The clinical severity of coronary artery disease was assessed on the basis of a modified score of Negri *et al.*[21]. Coronary circulation was divided into eight segments (see Fig. 1): left main coronary artery; proximal middle and distal segments of left anterior descending; proximal and distal right coronary and proximal and distal circumflex. Each segment was scored depending upon the severity of stenosis as follows: normal segment scoring 0, <50% stenosis — 1, 50–90% stenosis — 12·5, 91–99% stenosis — 20, 100% stenosis — 25 and the overall score was the sum of the 8 individual segments. The intra-observer correlation was 0·94.

The extent of atherosclerosis was assessed in two ways. Firstly a 'clinical vessel score' on a scale of 0–3 based on the system of Oberman *et al.*[22] was the number of vessels with a luminal diameter reduction of greater than 50%. The number of vessels involved was calculated as follows: right coronary artery = 1, circumflex = 1 and left anterior descending, first diagonal or both = 1. The intra-observer correlation was 0·82. This score enabled calculation of sensitivity and specificity of anti-mhsp65 as a diagnostic indicator of clinically significant atherosclerosis ($\geq$1 stenosis $\geq$50%). Secondly, a more detailed 'diffuseness score', as previous work has demonstrated greater correlations between risk factors and these scores than with the simpler vessel scores[23]. This score was based on a modification of the diffuseness score of Negri *et al.*[21] (see Fig. 1). The coronary circulation is divided into 15 segments and eight of these

are first order segments: proximal and middle right coronary artery, left main coronary artery, proximal middle and distal left anterior descending and proximal and distal circumflex. In addition, there were seven second order segments: distal right coronary artery, posterior descending branch (whether arising from circumflex or right coronary artery), obtuse marginal branch, posterolateral branch of circumflex and the first two diagonal branches of the left anterior descending. The first order segments received a score of 1 if there was any evidence of atherosclerosis, and the second order segments scored 0·5. The overall diffuseness score was the sum of the individual segment scores and the maximum score attainable was 11·5. The intra-observer correlation was 0·80.

Statistical analysis

In the first study, the anti-mhsp65 titres showed a strong positive skew, and so logarithmic data were used for all parametric methods. There were significant differences between assay batches and each log titre was corrected as far as possible by subtracting the mean log titre of the corresponding ELISA plate and adding the grand mean log titre. This left the mean log IgG anti-mhsp65 levels unchanged, i.e. left the geometric mean IgG anti-mhsp65 titre unchanged. Four of the other variables measured, anti-*Helicobacter pylori* titre, lipoprotein(a), triglycerides and CRP were approximately log-normally distributed and thus were expressed logarithmically before calculating confidence intervals. Correlations between continuous variables were examined using Spearman's rank correlation analysis. Relationships between continuous variables and categorical variables were assayed by Mann–Whitney and Kruskal–Wallis tests. Multiple linear regression analysis was used to test for residual relationships among interval variables while correcting for possible confounding influences. The Chi-square test was used to assess whether the presence of *H. pylori* infection was significantly associated with the presence of atherosclerosis. T-tests were used to compare the means of biochemical risk factors in *H. pylori* seropositive and seronegative subjects, before and after correcting for possible confounding influences. In the second study, the Kruskall–Wallis and Mann–Whitney Tests were used to examine for differences between the treatment and placebo groups.

Results

The correlations between the four coronary atherosclerosis scores and the titres of IgG anti-mhsp65 and IgG anti-*H. pylori* are demonstrated in Table 2. Correlations between continuous cardiovascular risk factors and anti-mhsp65 and anti-*H. pylori* titres are shown in Table 3. There were significant correlations between anti-mhsp65 titre and age (r = 0·24, $P = 0·005$) and possibly between titre and smoking as a continuous variable (r = 0·18, $P = 0·049$). There was no relationship between anti-mhsp65 titre and categorical variables of smoking or the other categorical variables of hypertension and family history of coronary artery disease. There was a significant correlation between anti-*H. pylori* titres and smoking as a continuous variable (r = 0·28, $P = 0·002$) and possibly between anti-*H. pylori* titre and fibrinogen (r = 0·20, $P = 0·031$). When the IgG anti-mhsp65 titre was corrected for the possible confounding influences of age and smoking and tested again for the relationship with atherosclerosis statistical significance was lost when comparing titre with severity or extent of disease. However, patients with atherosclerosis still had a significantly higher titre of anti-mhsp65 than those with no evidence of atherosclerosis (278·6 vs 171·0 U . ml^{-1}, $P = 0·012$). At best using a discriminant titre of anti-mhsp65 of 150 U . ml^{-1} this has 77·8% sensitivity but only 57·8% specificity for the detection of coronary atherosclerosis. When the anti-*H. pylori* titre was adjusted for the possible confounding influences of age, smoking and social class the relationship between titre and atherosclerosis lost statistical significance.

There was a moderate and highly statistically significant correlation between anti-mhsp65 titres and

Table 2 Spearman's rank correlations between severity and extent of coronary atherosclerosis and titres of antibodies to hsp65 and to* H. pylori *(before and after adjusting for confounders: age, social class and smoking consumption)

Atherosclerosis score	Correlation with anti-*H. pylori*	Correlation with IgG anti-mhsp65
Severity score	0·18 ($P = 0·040$)	0·21 ($P = 0·018$)
Vessel score	0·17 ($P = 0·036$)	0·18 ($P = 0·036$)
Clinical vessel score	0·12 ($P = 0·150$)	0·21 ($P = 0·012$)
Diffuseness score	0·22 ($P = 0·011$)	0·21 ($P = 0·016$)
Anti-*H. pylori*	—	0·39 ($P < 10^{-5}$)

Table 3 Spearman's rank correlations between continuous cardiovascular variables, anti-mhsp65 titres and anti* H. pylori *titres

Factor	Correlation with anti-mhsp65 titre	Correlation with anti-*H. pylori* titre
Age	0·24*	0·139
BMI	− 0·05	− 0·190
Social class	− 0·09	0·15***
Packets of cigarettes	0·18**	0·284*
Lipoprotein(a)	− 0·08	− 0·038
Triglycerides	− 0·02	− 0·038
Total cholesterol	− 0·05	− 0·055
Total/HDL cholesterol	− 0·22	− 0·064
Fibrinogen	− 0·10	0·196**

*$P < 0·01$, **$P < 0·05$, ***$P = 0·076$.

Table 4 Relationship between* H. pylori *seropositivity and continuous CVS risk factors before and after adjustment for confounders (age, smoking and social class)

	Before adjustment		After adjustment	
	H. pylori seronegative	*H. pylori* seropositive	*H. pylori* seronegative	*H. pylori* seropositive
Glucose (mmol . l^{-1})	5·75	5·76	5·71	5·77
Lipoprotein(a) (mg . dl^{-1})	22·39	21·87	22·91	21·88
Triglycerides (mmol . l^{-1})	1·58	1·68	1·62	1·66
Cholesterol (mmol . l^{-1})	5·75	5·55	5·64	5·63
LDL cholesterol (mmol . l^{-1})	3·82	3·58	3·66	3·68
HDL cholesterol (mmol . l^{-1})	0·87	0·91	0·91	0·89
Fibrinogen (mg . dl^{-1})	3·30	3·30	3·28	3·31
CRP (mg . l^{-1})	7·4	7·7	7·6	7·8
WBC ($\times 10^9 l^{-1}$)	6·52*	7·35*	7·08	7·14
Anti-mhsp65 (AU . ml^{-1})	187·5**	302·7**	251·2	263·0

*$P = 0·007$, **$P = 0·0008$.

antibody titre to *H. pylori* ($r = 0·39$, $P < 10^{-5}$). The correlations between these two titres was examined after adjusting for their possible common relationship with age, body mass index, smoking and social class and remained significant ($r = 0·38$, $P < 10^{-4}$).

In the group with no coronary atherosclerosis, 58% were seropositive for *H. pylori* compared with 71% of subjects with coronary atherosclerosis (difference not statistically significant). The relationship between *H. pylori* seropositivity and cardiovascular risk factors was examined (Table 4). *H. pylori* infection was not associated with raised concentrations of fibrinogen, cholesterol, triglycerides or glucose. However, it was associated with elevated titres of anti-mhsp65 ($P = 0·002$) and white blood cell count ($P = 0·006$). The significance of both was lost after adjustment for age, smoking history, body mass index and social class (Table 4).

In the second study there was no statistical difference in age, sex or pre-treatment anti-mhsp65 titres between placebo and treated groups. However with therapy the mean anti-mhsp65 level fell from 256·4 AU . ml^{-1} to 137·5 AU . ml^{-1} in the treatment group ($P = 0·033$) compared with a fall from 257·0 AU . ml^{-1} to 224·7 AU . ml^{-1} in the placebo group ($P =$ ns). Pre-therapy against post-therapy anti-hsp65 titres anti-mhsp65 titres for the treatment and placebo groups are illustrated in Figs 2 and 3, respectively.

Discussion

In the first study we show clear evidence that anti-mhsp65 antibodies are associated with angiographically detected coronary atherosclerosis, with statistically significant correlations between titre and severity or extent of disease (see Table 2). The statistical significance of the relationship between titre and presence of disease remained after adjustment for the possible confounding influences of smoking and age ($P = 0·012$). This is similar to the findings of Xu *et al.*[11] who in a study of carotid atherosclerosis, showed that an elevated titre of anti-mhsp65 correlated with the extent of atherosclerosis, but the correlation lost significance after adjustment for other risk factors. Again similarly to our results they did show that elevated titres of anti-mhsp65 was a risk factor for the presence of plaque, independent of other risk factors. However, anti-mhsp65 titre had insufficient sensitivity and specificity to be of use as a diagnostic marker of atherosclerosis, as had been hoped[7].

Titres of antibodies to *H. pylori* were shown to correlate with severity and extent of coronary atherosclerosis (Table 2). This supports other recent studies linking *H. pylori* infection with clinically manifest coronary artery disease[1–3]. In the present study and one previous study[4] the correlation was no longer significant after correction for age and social class. Previously it has been postulated that the association between *H. pylori* infection and ischaemic heart disease is due to systemic effects of *H. pylori* infection on fibrinogen concentration and total white blood cell count[3] but this has been challenged by a larger study[4]. Our own data showed no difference in fibrinogen levels between the seropositive and seronegative group (Table 4). However, the seropositive group clearly had elevated anti-mhsp65 titre and white cell count compared with the seronegative group (Table 4, prior to correction for age, body mass index, smoking and social class) supporting the possibility of an autoimmune process.

Titres of antibodies to mhsp65 and to *H. pylori* correlated highly significantly ($r = 0·39$, $P < 10^{-5}$) suggesting that *H. pylori* infection is an important influence on anti-mhsp65 titres. We further investigated the influence of *H. pylori* on anti-mhsp65 titres in the second study. We show evidence that eradication of *H. pylori* infection leads to a significant fall in anti-mhsp65 titres from 256·4 AU . ml^{-1} to 137·5 AU . ml^{-1} ($P = 0·033$, see Fig. 2).

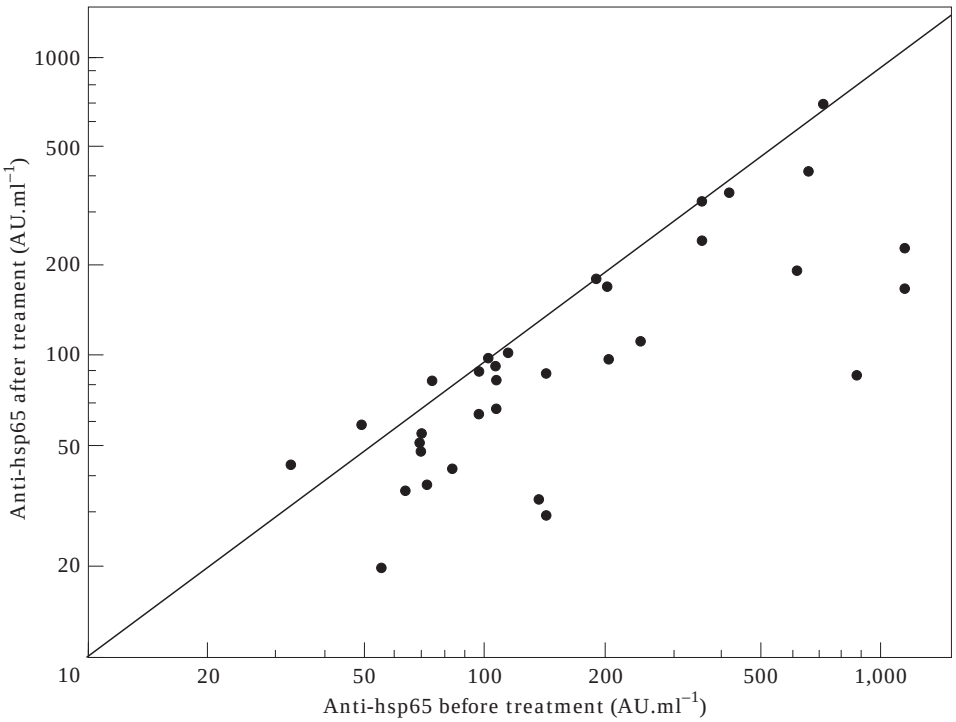

Figure 2 **Pre-therapy compared with post-therapy IgG anti-hsp65 titres in the group (n = 33) who had successful eradication of *H. pylori* infection.**

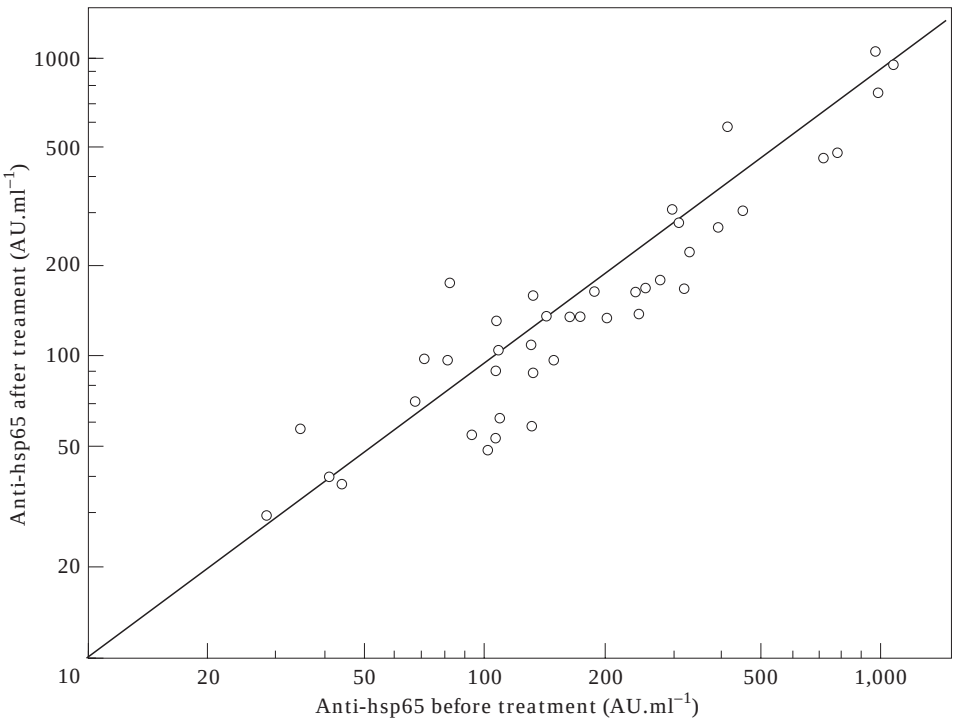

Figure 3 **Pre-placebo compared with post-placebo IgG anti-hsp65 titres in the group (n = 41) with confirmed persisting *H. pylori* infection.**

Our data suggests that *H. pylori* infection is a significant factor in the development of anti-mhsp65 titres, but presumably exposure to micro-organisms with cross-reacting hsp60/65 contributes to the titres as well. Certainly some of our data suggest this including most strongly from the second study where 7/33 (21·2%)

subjects who had successful eradication of *H. pylori* anti-mhsp65 titres stayed the same or rose. There is also supporting data from the first study including the fact that the correlation between antibodies to *H. pylori* and anti-hsp65 was no greater than 0·39 and in addition some subjects who were seronegative for *H. pylori* infection had significant titres of anti-mhsp65. *Chlamydia pneumoniae* infection has also recently been implicated in the pathogenesis of atherosclerosis by an unexplained mechanism[3,24]. The relationship remained after adjustment for cardiovascular risk factors including social class[3]. *C. pneumoniae* also expresses an hsp60 which shows close sequence homology with human counterparts[25]. Indeed another interpretation of our data is that the antibiotics given to eradicate *H. pylori* may have eradicated or decreased the load of other chronic bacterial infections and the reduction of these infections may be responsible to a greater or less extent for the fall in anti-mhsp65 titre. In support of this is our data in from the group who received antibiotics but subsequent breath tests indicated persistent *H. pylori* infection. In this cohort (n = 15) there was a trend for anti-mhsp65 titre to fall (from 103 AU ml^{-1} to 93·1 AU . ml^{-1}). It can at least be concluded that giving antibiotics leads to a fall in anti-mhsp65 titre thus indicating that bacterial infections are an important influence on anti-mhsp65 titre.

Thus an alternative hypothesis to explain the association between *H. pylori*, other bacterial infections and ischaemic heart disease based on the results of this current study, data of Xu *et al.*[8–12] and the known immunology of *H. pylori* can be summarized as follows: endogeneous hsp60 expression is induced on normal arterial intima by stresses such as hypertension and smoking. Exposure to *H. pylori* and other bacteria induces an immune response to bacterial hsp60/62/65 and the antibodies produced cross-react with the human hsp60. This could either initiate or contribute to the local inflammatory and auto-immune process in the arterial intima leading to initiation or worsening of the atherosclerotic lesion(s). Thus it may be the relative infecting load and extent of the immune response to infection that is important rather than just infection per se, and this may partly explain the contradictory results from studies[3,4] looking at seropositivity for *H. pylori* alone as a risk factor for ischaemic heart disease.

The relevance of the findings of this study to clinical practice is unclear. Certainly large scale prospective studies are required before we can say whether treatment of *H. pylori* and other infections and/or immuno-modulatory therapy can be prescribed to decrease cardiovascular risk. The potential clinical importance of this area was recently emphasized by the suggestion[26] that the inflammatory state of a coronary plaque might be a more important determinant of the clinical outcome than the degree of stenosis.

In summary therefore, we have demonstrated that anti-mhsp65 titres correlate with the severity and extent of coronary atherosclerosis and after correction for confounding factors, the elevated anti-mhsp65 titre remained statistically significantly associated with the presence of atherosclerosis ($P = 0·012$). However anti-mhsp65 titre had insufficient sensitivity and specificity to be of use as a diagnostic marker of atherosclerosis, as had been hoped[7]. *H. pylori* infection seems to be an important influence in anti-hsp65 titres as anti-hsp65 antibodies correlated with titres of antibodies to *H. pylori*. In addition we show that eradication of *H. pylori* infection leads to a significant fall ($P = 0·033$) in anti-mhsp65 titres. These data raise the possibility that exposure to *H. pylori* and other bacteria leads to increased risk of clinically manifest coronary artery disease by an auto-immune mechanism.

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