



Normal levels of inflammatory markers in treated patients with familial hypercholesterolaemia: a cross-sectional study

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DECLARATIONS

Competing interests

None of the authors has any competing interests. MS, DJB, SEH, PND, GRT and HAWN are present or past members of the Scientific Steering Committee of the Simon Broome Trust for Familial Hyperlipidaemia, in which capacity they act without remuneration

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Summary

Objective To assess the relationship of levels of inflammatory risk markers to presence of clinical coronary artery disease (CAD) in patients with treated heterozygous familial hypercholesterolaemia.

Design A cross-sectional study of patients on the Simon Broome Familial Hyperlipidaemia Register.

Setting Six hospital outpatient clinics in the UK.

Participants A total of 211 men and 199 women with heterozygous familial hypercholesterolaemia.

Main outcome measures Analysis of conventional risk factors and concentrations of high-sensitivity C-reactive protein (hsCRP), lipoprotein (a), serum intercellular adhesion molecule (sICAM), interleukin-6 (IL-6) and lipoprotein-associated phospholipase A₂ (LpPLA₂) mass.

Results CAD was present in 104 men and in 55 women; the mean ages of onset were 43.1 and 46.5 years, respectively. On univariate analysis there was a positive relationship of CAD with age, male sex, smoking, IL-6 and sICAM, and an inverse relationship with low-density lipoprotein (LDL) and LpPLA₂. On multivariate analysis, age, smoking, low LDL and low LpPLA₂ were associated with CAD. When LpPLA₂ values were adjusted for apoB and aspirin usage, there was no significant difference between those with and without CAD. Only age and smoking were independently associated with CAD in men, and IL-6 and lipoprotein(a) in women.

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Ethical approval

Ethical approval obtained by the six contributing lipid clinics

Guarantor

MS

Contributorship

DJB, PND, HAWN, SEH and MS contributed to the design and writing of the study. MC and NS supervised the laboratory analyses, JC undertook the statistical analysis and GRT contributed to the writing

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None

Conclusions Although on univariate analysis inflammatory marker levels were associated with CAD in these patients, the majority of the associations, including that for hsCRP, disappeared when corrected for smoking and apoB. This may be because atherosclerotic plaques in these statin-treated patients were quiescent or an effect of aspirin usage. In this observational study newer risk markers were not usefully associated with the presence or absence of symptomatic CAD.

Introduction

Familial hypercholesterolaemia (FH) is an autosomal-codominant disorder with an estimated frequency of one in 500 of the population. Most cases are caused by one of more than 1000 different mutations of the low-density lipoprotein (LDL) receptor¹ resulting in an accumulation of LDL cholesterol (LDL-C) in the plasma from birth and subsequent development of tendon xanthomata and atheroma.² In the heterozygous condition the cumulative risk of a coronary event by the age of 60 years without effective treatment is about 50% in men and 30% in women,^{3,4} with coronary disease occurring earlier in men than women⁵⁻⁷ and a marked increase occurring in women postmenopausally.⁶ The risk of a fatal coronary event is 100-fold higher than the standard mortality rate in untreated younger patients (<40 years old), although patients who survive through middle age no longer appear to be at substantially increased relative risk.^{8,9} Smoking is known to be a significant additional risk factor, but other factors influencing differences in individual susceptibility to coronary disease remain unclear.

Using data from the Simon Broome Register representing 26 years of observation, we have previously shown that life-expectancy has increased for patients diagnosed with heterozygous FH and treated with statins before the development of coronary artery disease (CAD).⁹ Although there is a strong intrafamily correlation with the age of coronary death in affected sibling pairs,¹⁰ relatives with identical LDL receptor mutations and similar LDL concentrations may have different outcomes.¹¹ This suggests that other genetic factors and/or environmental factors influence the susceptibility to coronary disease and might explain the wide variability in phenotypic expression. Few studies have assessed the role of

inflammatory risk markers; one such study showed no correlation between atherosclerosis and high-sensitivity C-reactive protein (hsCRP).¹² Our aim was to examine the association of conventional and inflammatory risk factors with documented clinical CAD in a large cohort of patients with treated xanthomatous FH.

Methods

A cross-sectional comparison was undertaken of white patients aged 18 years or more with treated heterozygous FH with and without clinically documented CAD. Eligible patients had been registered from 1980 onwards with the Simon Broome Familial Hyperlipidaemia Register by any one of six outpatient hospital lipid clinics. The diagnostic criteria for FH were defined as an untreated total cholesterol above 7.5 mmol/L or an LDL-C above 4.9 mmol/L, together with the presence of tendon xanthomata either in the patient or in a parent, child, grandparent, sibling, uncle or aunt.¹³ The names of registered patients had been flagged by the National Health Service Central Registry and, in the event of death, a copy of the death certificate was provided.

The clinical case-notes of all participants were scrutinized to confirm eligibility and to identify cases with documented CAD. Patients with known diabetes, renal or thyroid disorders were excluded. Patients were documented to have CAD if they had undergone a definite myocardial infarction (new Q waves and/or ST segment elevation and/or new T wave inversion persisting in more than two leads together with creatine kinase >400 IU/L or other equivalent enzyme changes), coronary artery bypass grafting or percutaneous transluminal coronary angioplasty or had angina with an ischaemic resting electrocardiogram (ECG) or an abnormal angiogram.

Excluded from the analyses were patients with acute coronary insufficiency, asymptomatic patients with an ischaemic ECG, those with a positive exercise test without positive angiography and those with a suspicious episode of acute chest pain or angina of effort diagnosed by a physician but with no significant ECG changes at rest or on exercise.

Participants remained on their usual drug therapy and attended the clinic after an overnight fast of at least 12 hours duration. Written consent was obtained. Measurements of blood pressure, height and weight were made. Medication, including presence or absence of statin therapy and its duration, and antiplatelet (aspirin) therapy was recorded. Nicotinic acid had not been prescribed for any of the patients. Alcohol consumption and smoking were documented (ever smoking was defined as having smoked at least 1 cigarette a day for at least 1 year), and a venous blood sample was taken. Additional clinical and demographic information was obtained from the registration form completed on enrolment in the Simon Broome Register. The study received approval from the local ethics committee of each of the six participating centres.

Biochemical measurements

Venous blood specimens were collected into EDTA, fluoride and citrate vacutainers. Except for a sample collected into EDTA for haematological measurements, all were centrifuged immediately to separate plasma for the measurement of lipids, lipoproteins, apolipoproteins and lipoprotein(a) (Lp(a)) by the Department of Chemical Pathology, University College Hospital, London. Plasma total and high-density lipoprotein (HDL) cholesterol, triglyceride and glucose were measured using standard enzymatic methods (Roche Diagnostics, Welwyn Garden City, UK). ApoA1, apo B and Lp(a) were measured by immunoturbidimetry on a Cobas-Bioanalyser (Roche Diagnostics), with kits obtained from DiaSorin using SPQ SPQ II test systems and calibrators (DiaSorin Ltd., Wokingham, UK). Plasma samples for all other measurements were stored at -85°C until analysed. Plasma adiponectin, serum intercellular adhesion molecule (sICAM) and interleukin-6 (IL-6) concentrations were

determined using enzyme-linked immunosorbent assays (R&D Systems Europe Ltd., Abingdon, UK) with detection limits of 0.9, 0.3 and 1.0 ng/L, respectively; intra-assay coefficients of variation of 6.8%, 8.7% and 5.8%, respectively; and inter-assay coefficients of variation of 10.2%, 9.1% and 12.4%, respectively. hsCRP was assayed by latex particle-enhanced immunonephelometry using the BN ProSpec System (Dade-Behring UK Ltd., Milton Keynes, UK). The hsCRP detection limit was 0.12 mg/L and the intra- and inter-assay coefficients of variation were 5.9% and 6.1%, respectively. Lipoprotein-associated phospholipase A₂ mass (LpPLA₂) was measured with an enzyme-linked immunoassay using PLACII (Diadexus, San Francisco, CA, USA).¹⁴ The range of detection was 1.3–1000 ng/mL and the intra-assay and inter-assay coefficients of variation were 4% and 10%, respectively. There was no cross-reactivity with other A₂ phospholipases.

Statistical analysis

Variables were transformed where appropriate (log or square root transformation) to give normal distributions. Adjusted mean values were predicted from linear regression models including age, smoking status, gender, medication use and CAD status. Odds ratios (ORs) for CAD were also obtained from logistic regression models, both unadjusted and including age, smoking status and gender as covariates. For the continuous variables, ORs are shown for a one standard deviation (SD) increase in the variable. For Lp(a) the top tertile was compared with the bottom two tertiles. Stepwise logistic regression analysis using backwards selection including the above covariates was performed to establish independent associations with risk. It has been shown that variable selection methods (stepwise models) are unstable and frequently identify spurious noise variables as independent predictors. For this reason, the stepwise models were validated using repeated random sampling from the original data-set allowing individuals to be included more than once in each sample. The stepwise model was run on each of these samples. Variables were only included in the final model if they were consistently identified as predictors in these alternate samples. Variables included in the original

Table 1
Characteristics of participants by CAD status and sex

Variable	Men		Women	
	CAD- n = 107	CAD+ n = 104	CAD- n = 144	CAD+ n = 55
CAD (%)		49.3		27.6
Mean age in years (SD) at entry to study	44.2 (12.5)	56.0 (10.2)	44.8 (14.4)	56.6 (10.4)
Mean age in years (SD) at first CAD event		43.1 (9.8)		46.5 (11.9)
Ever cigarette smoker (%)	35.5	62.1	39.6	58.2
Current cigarette smoker (%)	12.1	10.7	17.4	12.7
Mean BMI in kg/m ² (SD)	24.1 (4.2)	25.4 (3.2)	23.6 (4.1)	25.0 (4.1)
Statin therapy (%)	92.5	99.0	74.3	94.5
Median duration of statin therapy in years (IQR)	5.0 (2.0, 6.7)	7.0 (5.2, 8.3)	4.0 (1.8, 6.4)	7.2 (5.2, 8.6)
Antiplatelet therapy (%)	19.0	83.0	10.9	73.1
Total cholesterol (mmol/L)	6.54 (1.06)	6.21 (1.31)	7.14 (1.40)	6.72 (1.28)
LDL (mmol/L)	4.58 (1.02)	4.14 (1.18)	4.92 (1.41)	4.45 (1.33)
HDL (mmol/L)	1.23 (0.28)	1.24 (0.37)	1.51 (0.36)	1.37 (0.32)
Triglycerides (mmol/L)	1.27 (0.57)	1.44 (0.66)	1.22 (0.53)	1.45 (0.70)
Apolipoprotein A1 (g/L)	1.27 (0.25)	1.34 (0.33)	1.45 (0.32)	1.43 (0.34)
Apolipoprotein B (g/L)	1.23 (0.34)	1.17 (0.37)	1.34 (0.35)	1.33 (0.43)

IQR, interquartile range; BMI, body mass index

model all occurred frequently (>60 selections) in the bootstrap samples, and no other important variable was identified, confirming stability of the original model.¹⁵

Results

A total of 458 patients with FH were enrolled in the study; 48 patients (10.5%) with possible CAD were subsequently excluded because they failed to meet the prespecified diagnostic criteria for CAD. Data are therefore presented for 410 patients.

Table 1 shows the characteristics of the participants. The 22% difference in prevalence of CAD between men and women was significant (95% confidence interval [CI], 13–31%; $P < 0.001$). The age at diagnosis of CAD was earlier in men than in women, and in patients with a history of smoking compared with those who had never smoked (3.8 years; 95% CI, 0.48–7.2; $P = 0.025$); this difference did not differ significantly between men and women. A history of smoking was also significantly associated with CAD (OR, 2.5). Most patients with CAD were treated with simvastatin or atorvastatin 40 mg daily, whereas

over 25% of women without CAD were not on a statin.

Table 2 shows concentrations and statistical analysis of potential risk factors in our patients. On univariate analysis there were significant associations of sICAM, IL-6 and lower LpPLA2 with CAD status in men and women combined; after adjustment for age, gender and smoking only, LpPLA2 showed a significant and inverse association. However, when LpPLA2 levels were adjusted for serum apoB concentration and use of aspirin, the association with CAD was no longer significant (OR, 0.86; CI, 0.64–1.17; $P = 0.33$).

Multivariate analysis using a stepwise model for both sexes combined, and including all variables, identified age, ever smoking, low HDL (OR, 0.69; CI, 0.54–0.87; $P = 0.002$) and low LpPLA2 (OR, 0.74; CI, 0.58–0.94; $P = 0.01$) as independent predictors of CAD. Separate analysis by sex revealed only age and smoking to be significant in men, while in women age, IL-6 (OR, 2.33; CI, 1.27–4.29; $P = 0.007$) and Lp(a) (OR, 2.94; CI, 1.14–7.60; $P = 0.03$) were positively associated, and HDL (OR, 0.53; CI, 0.32–0.90; $P = 0.02$) and hsCRP (OR, 0.56; CI, 0.32–0.99; $P = 0.05$) were inversely associated with CAD.

Table 2
Geometric means and approximate SDs for risk factors by CAD status in men and women separately and together; adjusted mean values predicted from fitted regression models for patients with and without CAD; ORs for CAD for a one SD increase in each variable with their 95% CI

	Geometric mean (approximate SD)		Men and women					
	Men	Women	Geometric mean (approximate SD)	Unadjusted OR (95% CI)	P value	Adjusted means (SE)	Adjusted OR (95% CI)	P value
hsCRP (mg/L)	CAD- 0.94 (1.03) CAD+ 1.45 (1.74)	1.44 (1.63) 1.28 (1.30)	1.19 (1.35) 1.39 (1.58)	1.15 (0.93-1.41)	0.20	0.95 (0.10) 0.97 (0.12)	0.96 (0.75-1.24)	0.77
sICAM (ng/mL)	CAD- 285.4 (105.0) CAD+ 305.8 (93.5)	283.2 (99.5) 324.0 (113.6)	284.2 (101.7) 312.0 (100.5)	1.32 (1.07-1.62)	0.01	281.5 (9.6) 304.9 (11.6)	1.23 (0.97-1.55)	0.08
IL-6 (pg/mL)	CAD- 1.66 (1.14) CAD+ 2.28 (1.54)	1.42 (1.04) 2.01 (1.11)	1.52 (1.09) 2.19 (1.39)	1.72 (1.38-2.15)	<0.0001	1.66 (0.11) 1.93 (0.14)	1.23 (0.95-1.60)	0.11
Adiponectin ¹ (µg/mL)	CAD- 9.24 (7.32) CAD+ 8.65 (6.78)	12.46 (8.31) 15.46 (9.54)	10.99 (8.03) 10.77 (8.28)	0.97 (0.79-1.19)	0.79	9.49 (0.79) 9.26 (0.89)	0.95 (0.71-1.27)	0.74
Lp(a) (g/L) ²	CAD- 0.33 [0.15-0.58] CAD+ 0.30 [0.14-0.51]	0.27 [0.14-0.5] 0.44 [0.19-0.65]	0.29 [0.14-0.56] 0.37 [0.16-0.62]	1.27 (0.77-2.11)	0.35	-	1.51 (0.84-2.70)	0.17
LpPLA2 (ng/mL)	CAD- 357.8 (157.4) CAD+ 325.6 (114.1)	375.8 (150.8) 321.2 (112.0)	367.8 (153.9) 324.1 (113.1)	0.71 (0.57-0.89)	0.002	360.7 (13.7) 324.1 (14.3)	0.76 (0.59-0.96)	0.02

BMI, body mass index

This table is adjusted for age, gender and smoking

¹Mean and SD transformed back from square root transformed data, adjusted for age, gender, smoking and BMI

²Median (IQR). OR is for top tertile compared with bottom two tertiles (cut-off 0.66)

Table 3**Partial correlations between conventional and inflammatory risk markers, with variables adjusted for age, sex, smoking, CAD status and BMI**

Men and women	LpPLA2		hsCRP		IL-6		Adiponectin		sICAM	
	r	P value	r	P value	r	P value	r	P value	r	P value
Total cholesterol	0.24	<0.0001	0.14	0.02	0.07	0.20	0.14	0.01	-0.03	0.39
LDL	0.25	<0.0001	0.12	0.04	0.13	0.03	0.12	0.04	0.01	0.84
HDL	0.01	0.87	-0.17	0.004	-0.25	<0.0001	0.30	<0.0001	-0.19	0.001
Triglyceride	0.02	0.71	0.29	<0.0001	0.12	0.04	-0.20	<0.0001	0.05	0.38
ApoA1	-0.10	0.07	-0.11	0.05	-0.14	0.02	0.16	0.005	-0.05	0.35
apoB	0.22	<0.0001	0.16	0.005	0.08	0.18	-0.04	0.50	0.01	0.82
hsCRP	0.09	0.13	-	-	-	-	-	-	-	-
IL-6	-0.004	0.94	0.40	<0.0001	-	-	-	-	-	-
Adiponectin	0.06	0.30	-0.11	0.07	-0.02	0.71	-	-	-	-
sICAM	-0.25	<0.0001	0.20	0.001	0.29	<0.0001	0.16	0.007	-	-
Lp(a)	0.18	0.008	-0.06	0.39	0.10	0.16	-0.07	0.34	-0.004	0.95

BMI, body mass index

Table 3 shows that there were numerous correlations between traditional and inflammatory risk markers. For example, hsCRP levels were positively correlated with total cholesterol, LDL, triglyceride, apoB, IL-6 and sICAM, and inversely with HDL and apoA1; LpPLA2 levels were positively correlated with total cholesterol, LDL, apoB and Lp(a).

The relationship between smoking and inflammatory risk factors was also explored. There was a strong relationship between smoking and mean hsCRP levels (1.55; SD, 1.73 in ever smokers versus 1.06; SD, 1.19 in never smokers; $P = 0.001$) and between smoking and mean IL-6 levels (1.96; SD, 1.40 in ever smokers versus 1.58; SD, 1.09, in never smokers; $P = 0.003$).

Effect of the mutation causing FH

A total of 396 patients were examined for presence or type of mutation: in 81 no mutation was identified; 309 had a mutation in the LDL receptor gene; 12 had a mutation in apoB; and seven had a mutation in PCSK9. Geometric means of adiponectin levels and LpPLA2 mass, after adjustment for age, sex, smoking, apoB and antiplatelet drug use, were significantly higher in patients in whom a causal mutation for FH had been identified than in those with no known mutation (11.38 versus 9.08 $\mu\text{g}/\text{mL}$, $P = 0.01$ and 358.9

versus 314.6 ng/mL , $P = 0.02$). There were no significant differences in inflammatory markers according to which gene was mutated, nor was mutation status a significant determinant of the presence or absence of CAD after adjustment for age, sex, smoking, apoB and antiplatelet drug use.

Discussion

In this cohort of FH patients, we have previously examined a wide spectrum of established and emerging coronary risk factors including total, LDL and HDL cholesterol, triglyceride, apoA1, apoB, homocysteine, fibrinogen, plasminogen activator inhibitor-1, white blood count (WBC), haematocrit, glucose, systolic and diastolic blood pressure.¹⁶ Although age, sex, smoking and low HDL were significantly associated with the presence of CAD, on multivariate analysis there was no evidence to suggest that any of the other above-mentioned coronary risk factors were associated with CAD. The purpose of this present analysis was to examine the same patients whether there was any residual risk attributable to inflammatory markers which might require novel therapies. The main finding in the present study was that none of these markers were associated with the presence of established CAD, although when women

were analysed separately, IL-6 and Lp(a) were found to be independently associated with the latter.

The advantage of our study is that it comprised a large number of well-documented patients with FH. The disadvantages are firstly that the design is cross-sectional, and secondly that our patients were survivors of their coronary events. Our study, which is retrospective, is unable to show whether prospective measurement of inflammatory markers in individuals with FH might indicate those at increased risk of an event. On the contrary it shows that patients with FH on long-term statin therapy do not have high levels of inflammatory markers, suggesting stabilization of atherosclerosis and decreased risk of further CAD events. This explanation is in accordance with the decreased risk associated with LDL-lowering treatment in the Dutch and UK Simon Broome long-term cohort studies of FH patients.^{8,9}

Other studies also suggest that the role of inflammatory markers is controversial. For example, in patients with untreated FH no correlation was shown between hsCRP and WBC and carotid intima media thickness.¹² In a four-year follow-up study to examine the predictive power of LpPLA2 for future cardiovascular events in non-FH patients with CAD, it was shown that LpPLA2 did not significantly predict events when adjusted for LDL.¹⁷ Again in contrast to the situation in patients with FH, the Atherosclerosis Risk in Communities (ARIC) study showed that LpPLA2 was predictive of CAD risk only in those with low LDL levels.¹⁸ The Bruneck study (765 subjects with a follow-up of 10 years) identified oxidized phospholipid//apoB and Lp(a) as risk factors; CAD risk was increased with increasing LpPLA2 activity, the latter being influenced by LDL and apoB as well as by insulin-resistance and ferritin levels.¹⁹ The importance of adjusting LpPLA2 for lipid levels was again emphasized in a population-based cohort study from Malmö which showed LpPLA2 to be associated with stroke, but when adjusted for LDL levels, not with CAD events.²⁰

In our study LpPLA2 mass, 85% of which is carried in the circulation by apoB-containing particles, mainly LDL, was lower in men and women with a history of CAD than in those without CAD (Table 2). The influence of apoB concentration on

LpPLA2 mass in patients on statins was described by the Heart Protection Study Collaborative Group²¹ and the differences in LpPLA2 we observed in patients with and without CAD presumably reflect the LDL-lowering effect of statin therapy as a confounding factor. In addition, the use of aspirin was strikingly greater in patients with CAD (Table 1); after adjustment for this and apoB levels LpPLA2 was no longer significantly lower in the CAD-positive group. In keeping with this, a study that looked at potential modifiers of LpPLA2 concentration found an inverse relation between LpLPA2 levels and use of cholesterol-lowering medication.²²

Data from both Dutch⁸ and UK⁹ studies have shown that the relative risk of CAD in FH subjects after the age of 60 is not significantly higher than in the general population and is much reduced as compared with the high risk at a younger age, possibly reflecting stabilization of coronary lesions by lipid-lowering therapy. In our study the mean interval between CAD events and the blood sampling was 13 years in men and 10 years in women. This shows that measuring markers of acute inflammation such as hsCRP and LpPLA2 in treated subjects long after the event is unlikely to differentiate between those with and without a past CAD event. In contrast high levels of Lp(a), which are not affected by statins, aspirin or age, were associated with CAD in women in the present study. This is consistent with data from the Emerging Risk Factors Collaboration.²³ It is possible that Lp(a) did not emerge as a significant risk factor for men because of the size of our study; neither did we measure apo(a) gene size, unlike the PROCARDIS which has clearly shown that it is the apo(a) gene, *LPA*, as well as Lp(a) level, which is associated with cardiovascular events.²⁴ The hypothesis that lowering Lp(a) will reduce cardiovascular events may be investigated in two studies, AIMHIGH and HPS2-THRIVE, both trials involving nicotinic acid compounds, although the former is now terminated. In our study of treated patients hsCRP was not independently associated with CAD, and data from large Mendelian randomization studies suggest that elevated hsCRP is not causative of cardiovascular disease, although inflammation may be contributory.²⁵

In 2003, the Conference on 'Markers of Inflammation and Cardiovascular Disease' concluded

that 'many of these markers (including inflammatory markers) are not yet considered applicable for routine risk assessment because of: (1) lack of measurement standardization; (2) lack of consistency in epidemiological findings from prospective studies with endpoints; and (3) lack of evidence that the novel marker adds to risk prediction over and above that already achievable through the use of established risk factors'.²⁶ Not all studies on the contribution of contemporary biomarkers to the prediction of cardiovascular risk have been positive. There were only small increases in the ability to classify risk as measured by the C-statistic in the Framingham Heart Study follow-up.²⁷ More recently a report from the College of American Pathologists concluded that LpPLA2 was more relevant to acute stroke than to CAD events.²⁸ Its usefulness as a risk marker in patients on statins, as were most of those in our study, is constrained by its intrinsic association with LDL and apoB levels.

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