Clinical Science (2009) **116**, 53–60 (Printed in Great Britain) doi:10.1042/CS20070404

comment

Association between both lipid and protein oxidation and the risk of fatal or non-fatal coronary heart disease in a human population

Mark WOODWARD*†, Kevin D. CROFT‡, Trevor A. MORI‡, Henrietta HEADLAM‡, Xiao Suo WANG§, Cacang SUARNA§‖, Mark J. RAFTERY¶, Stephen W. MacMAHON† and Roland STOCKER§‖

*Department of Medicine, Mount Sinai Medical Center, New York, NY 10029-6574, U.S.A., †The George Institute for International Health, University of Sydney, Camperdown, NSW 2050, Australia, ‡School of Medicine and Pharmacology, University of Western Australia, Perth, WA 6001, Australia, §School of Medical Sciences, University of New South Wales, Sydney, NSW 2052, Australia, ||School of Medical Sciences, Bosch Institute, Medical Foundation, University of Sydney, Camperdown, NSW 2050, Australia, and ¶Biomedical Mass Spectrometry Unit, University of New South Wales, Sydney, NSW 2052, Australia

A B S T R A C T

The role of oxidative damage in the aetiology of coronary disease remains controversial, as clinical trials investigating the effect of antioxidants have not generally been positive. In the present study, 227 coronary cases, identified from a cohort study, were matched, by age and gender, with 420 controls in a nested case-control design. Stored plasma samples were analysed for F_2-isoprostanes by stable isotope dilution MS, and specifically oxidized forms of apoA-I (apolipoprotein A-I) by HPLC of HDL (high-density lipoprotein). Median values of F_2-isoprostanes were higher in plasma samples that contained oxidized apoA-I compared with samples with undetectable oxidized apoA-I (1542 compared with 1165 pmol/l). F_2-Isoprostanes were significantly correlated with variants of non-oxidized apoA-II ($r = -0.15$) and were associated with HDL-cholesterol ($P < 0.0001$). F_2-Isoprostanes in cases (median, 1146 pmol/l) were not different from controls (1250 pmol/l); the odds ratio (95% confidence interval) for a 1 S.D. increase in F_2-isoprostanes was 1.08 (0.91–1.29). Similarly, there was no independent association between the presence of oxidized apoA-I, detected in approx. 20% of the samples, and coronary risk. In conclusion, we found no evidence of associations between markers of lipid (F_2-isoprostanes) and protein (oxidized apoA-I) oxidation and the risk of fatal or non-fatal coronary heart disease in a general population. This may be due to a true lack of association or insufficient power.

INTRODUCTION

HDL (high-density lipoprotein) is thought to protect against CHD (coronary heart disease) by promoting reverse cholesterol transport and by antioxidant, anti-inflammatory and antithrombotic effects [1]. HDL may aid the detoxification of pro-atherogenic oxidized lipids [2]. CETP (cholesteryl ester transfer protein) transfers oxidized lipids from LDL (low-density lipoprotein) to HDL [3], and the apolipoproteins of HDL reduce lipid hydroperoxides to the corresponding alcohols [4], thereby decreasing the likelihood of additional oxidation

Key words: antioxidant, apolipoprotein, coronary heart disease, isoprostane, lipid.

Abbreviations: apoA, apolipoprotein A; BMI, body mass index; CETP, cholesteryl ester transfer protein; CHD, coronary heart disease; CI, confidence interval; CRP, C-reactive protein; HDL, high-density lipoprotein; IL-6, interleukin-6; LDL, low-density lipoprotein; MetO, methionine sulfoxide; MS/MS, tandem MS; OR, odds ratio.

Correspondence: Professor Mark Woodward (email mark.woodward@mountsinai.org).

reactions. In addition, oxidized lipids associated with circulating HDL are rapidly removed by the liver [5,6].

HDL is also subject to oxidation. *In vitro*, HDL lipids are oxidized in preference to those of LDL [2]. In human atherosclerotic lesions, HDL lipids are oxidized to an extent comparable with that of LDL [7,8]. Such oxidized HDL could conceivably re-enter the circulation, as HDL is smaller than LDL and is comparatively less retained via interaction with extracellular matrix in the vessel wall. Indeed, there is evidence for the presence of oxidized HDL in the circulation of subjects with atherosclerotic disease [9–11]. ApoA-I (apolipoprotein A-I) and apoA-II are the major proteins of HDL. HDL can chemically reduce lipid hydroperoxides in a reaction accompanied by the conversion of specific methionine residues of apoA-I and apoA-II into MetO (methionine sulfoxide) [12,13]. Similarly, and independent of the nature of the oxidant involved, apoA-I_{+16} (apoA-I containing $MetO^{86}$ or $MetO^{112}$ with an increase in mass of 16 Da), apoA-I_{+32} (containing $MetO^{86}$ and $MetO^{112}$ with an increase in mass of 32 Da) and apoA-II_{+16} accumulate as HDL lipids become peroxidized [14]. Therefore these specific forms of oxidized apoA-I and apoA-II may be useful markers of *in vivo* HDL oxidation and, hence, possibly atherosclerosis [14].

F_2-isoprostanes are stable secondary oxidation products of arachidonic acid commonly used as markers of *in vivo* lipid oxidation [15]. There is good evidence from animal models of free-radical injury and human conditions associated with increased oxidative stress that, when determined in plasma or urine by suitable MS methods, F_2-isoprostanes provide a reliable measure of *in vivo* oxidative stress.

To date, few prospective epidemiological studies of CHD have considered the diagnostic value of markers of oxidative damage. In the present study, we used samples from a nested case-control study to investigate the association between CHD and markers of protein (apoA-I_{+16}, apoA-II_{+16} and apoA-I_{+32}) and lipid oxidation (F_2-isoprostanes) in a general population.

MATERIALS AND METHODS

Subjects

The parent study for the nested study was the Fletcher Challenge Cohort Study in New Zealand, a sample from a general population, including those with and without pre-existing CHD [16,17]. Between June 1992 and March 1993, samples of EDTA plasma (2 ml) were taken and stored at $-70\,^{\circ}C$ in the presence of butylated hydroxytoluene (22 μg/ml). Prospective follow-up of the 10529 subjects used national record linkage systems. CHD at baseline was positive if the respondent answered positively to the question 'Have you ever been told by a doctor that you have had a heart attack?'. CHD during follow-up was defined as a fatal coronary event or non-fatal hospital-diagnosed myocardial infarction (ICD-9 codes 410–414). For the nested study, cases were all members of the study who had CHD during the follow-up period (median, 5.6 years). In total, 227 cases were identified. After allowing for problems later found with stored samples, for each case between one and three (mean, 1.9) matched controls were randomly sampled; anyone who became a case after the date of the index coronary event of the case was eligible for selection as a matched control, and controls could be matched with more than one case [18]. Matching variables were age, gender and study (occupational or community samples). In total, 420 unique subjects without CHD ('unique controls') were identified. Age was matched, to the index case, within 1 year for all but three controls, these being matched within 5 years.

Patients provided informed consent and ethics approval was provided by the University of Sydney.

Assays

Frozen aliquots of plasma samples were shipped, on dry ice, to laboratories in Perth (Australia) for analyses of F_2-isoprostanes, Sydney (Australia) for analyses of apolipoproteins and Glasgow (Scotland, U.K.) for analyses of haemostatic and inflammatory biomarkers [17]. All samples were thawed once only.

Native and oxidized apoA-I and apoA-II

Immediately after thawing, HDL was isolated from plasma by ultracentrifugation and then subjected to HPLC for determination of native and oxidized apoA-I and apoA-II [19]. Native apoA-I eluted at approx. 31 min as a single species, whereas apoA-II (dimer) eluted at approx. 40 min as three distinct species [14], hereafter referred to as apoA-II-1, apoA-II-2 and apoA-II-3. Each of these apoA-II species was purified further by HPLC and characterized as dimers, monomers and trypsin peptides by time-of-flight MS, with scans acquired for m/z 250–2000. Precursor ions corresponding to the expected N- or C-terminal peptide ions were selected by Q1 for subsequent MS/MS (tandem MS) analysis. Time-of-flight mass spectra were analysed, and individual protein or peptide masses calculated using Bayesian reconstruct algorithms (Applied Biosystems). MS/MS spectra were compared and interpreted manually.

The HPLC method separated three species of oxidized apoA-I: apoA-I_{+32}, apoA-I_{+16} containing $MetO^{112}$ and apoA-I_{+16} containing $MetO^{86}$ that eluted at approx. 23, 24 and 30 min respectively. Oxidized apoA-II (i.e. apoA-II_{+16}) eluted as three species at approx. 36 min. All apolipoproteins were quantified by peak area comparison with standards prepared by HPLC and chemically verified for authenticity by electrospray ionization MS [12]. The proportion of apoA-I or apoA-II present in oxidized forms was expressed as a percentage of the

corresponding total apolipoprotein. Intra- and inter-assay variations for all oxidized apolipoproteins were comparable and, for example, were 5.2 and 9.3 % for apoA-I_{+32} respectively.

F_2-Isoprostanes

F_2-Isoprostanes were measured using a method published previously [20], with minor modification. In brief, 15-F_{2t}-IsoP-d_4 and 8-F_{2t}-IsoP-d_4 (5 ng) were added as internal standards to plasma (250 μl). After base hydrolysis with KOH (1 mol/l), samples were diluted with 2 ml of 0.1 mol/l sodium acetate solution (pH 4.6) and purified by solid-phase extraction on a mixed reverse-phase/anion-exchange column (Bond Elut-Certify II; Varian). F_2-Isoprostanes were eluted with ethyl acetate/methanol (9:1, v/v), dried under vacuum and derivatized to the trimethylsilyl pentafluorobenzylesters and analysed by GC/MS on an Agilent 6890 gas chromatograph coupled to an Agilent 5973 mass selective detector using electron capture negative ionization. F_2-Isoprostanes were detected by selected ion monitoring using *m/z* 569 and *m/z* 573 for 15-F_2-IsoP, and 15-F_{2t}-IsoP-d_4 and 8-F_{2t}-IsoP-d_4 respectively. The intra- and inter-assay variations for plasma F2-isoprostanes were 5.6 and 8.0 % respectively.

Other variables

Fibrinogen and high-sensitivity CRP (C-reactive protein) were assayed using automated immunonephelometric assays (Dade Behring) [17], and IL-6 (interleukin-6) was assayed using a high-sensitivity ELISA (R&D Systems) [17].

Statistical methods

Cases and unique controls were compared, for continuous variables, using medians and extreme values. Associations between markers of antioxidant stress and coronary risk factors were assessed, adjusting for age and gender, using general linear models after transforming the markers of antioxidant stress to approximate normality. Spearman rank correlations were used to assess associations between the continuous measures of oxidative stress. ORs (odds ratios) for CHD (fatal or non-fatal) were estimated from conditional logistic regression models [17] using the matched sets as units of observation. ORs were also obtained separately for subjects without CHD at baseline.

RESULTS

Owing to limited plasma samples, some results were incomplete. Apolipoprotein variables and F_2-isoprostanes were measured on 205 and 227 of the 227 cases and on 334 and 417 of the 420 unique controls respectively.

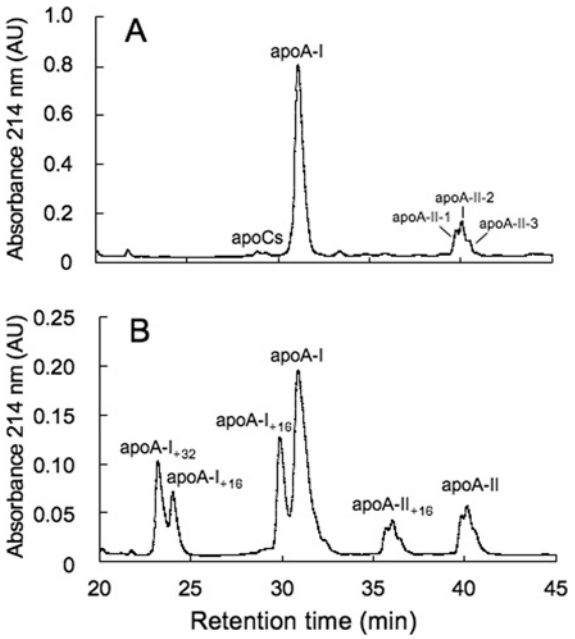

Figure 1 Representative HPLC traces of native (non-oxidized) HDL (A) and HDL oxidized *in vitro* by exposure to the peroxyl radical generator 2,2′-azo-bis(2-amidinopropane) dihydrochloride (B)

AU, arbitrary units.

Confirmation of native and oxidized forms of apoA-I and apoA-II

ApoA-I and apoA-II were well separated (Figure 1A). The masses determined for purified apoA-II-1, apoA-II-2 and apoA-II-3 were 17380.6, 17252.6 and 17124.8 Da respectively. Further analyses of freshly isolated apoA-II-1 and apoA-II-2 showed that they contained species corresponding to full-length dimeric apoA-II without and with one or two glutamine residue(s) lost, and dimeric apoA-II with two glutamine residues and one threonine residue lost respectively (Table 1). Chemical reduction of apoA-II-1 and apoA-II-2 produced monomers with masses of 8706, 8758 and 8477 Da, corresponding to monomeric apoA-II, apoA-II minus glutamine and apoA-II minus glutamine and threonine respectively. Analysis of partial tryptic digests and sequences of these digests confirmed and unambiguously identified apoA-II-1 and apoA-II-2 as apoA-II isomers with the C-terminal glutamine residue and glutamine plus threonine residues lost respectively (Table 1).

A typical chromatogram obtained from peroxyl radical-oxidized HDL is shown in Figure 1(B). ApoA-I contains three methionine residues, of which Met^{86} and Met^{112} are converted into MetO during oxidation of HDL [10,12]. MS analyses confirmed the chemical nature of apoA-I_{+16} and apoA-I_{+32}, and that apoA-II_{+16} contained one of the two Met^{26} residues of the dimer as MetO. Overall, the results obtained with native and peroxyl

Table 1 MS analysis of apoA-II isomers

ApoA-II-Q, apoA-II-2Q and apoA-II-Q&T refer to apoA-II with one glutamine residue, two glutamine residues and one glutamine residue plus threonine residue lost respectively. *Fragmentation spectra of $[M+3H]^{3+}$ precursor ions were consistent with sequences.

	Mass from MS (Da)		
Compound	ApoA-II-1	ApoA-II-2	Theoretical mass (Da)
ApoA-II dimer	17411	—	17414
ApoA-II dimer-Q	17283	17283	17286
ApoA-II dimer-2Q	—	17155	17158
ApoA-II monomer	8706	8707	8708
ApoA-II monomer-Q	8578	8578	8580
ApoA-II monomer-Q&T	—	8477	8479
ApoA-II_{1-28}	3235.8	3235.6	3235.5
ApoA-II_{56-77}	2385.1*	2385.2*	2382.2
ApoA-II_{56-76}	2257.0*	2257.6*	2257.1
ApoA-II_{56-75}	—	2155.6*	2156.1

radical-oxidized HDL were consistent with previous studies [12–14]. Such formation of apoA-I_{+16} and apoA-I_{+32} also occurs when HDL is oxidized with lipoxygenase [10] or myeloperoxidase/H_2O_2/tyrosine residue (results not shown).

Presence of oxidized apolipoproteins in HDL

All 534 HDL samples isolated contained apoA-I and apoA-II. Of these, 116 (22 %) also contained apoA-I_{+16} (Figure 2) at 3–45 % of the corresponding apoA-I content. ApoA-I_{+16} was present exclusively as $MetO^{86}$. Only nine (1.7 %) of the samples contained apoA-I_{+32} at 22–77 % of the corresponding apoA-I content. ApoA-I_{+16} was detected in only three of these apoA-I_{+32}-containing samples and, in each instance, it was apoA-I_{+16} that contained $MetO^{86}$ (results not shown).

Presence of oxidized lipids in plasma

Of the biochemical markers, only F_2-isoprostanes, apoA-II-1 and apoA-II-2 had a continuous distribution; the remaining variables had few non-zero percentages and were thus analysed as dichotomous variables (zero/non-zero percentage). There were large differences between the cases and controls in the summary values of coronary risk factors, with total cholesterol, triacylglycerols (triglycerides), fibrinogen, percentage smoking, IL-6 and CRP all having considerably higher medians, and HDL lower, in the cases (Table 2). In contrast, there was little difference between cases and controls for any of the oxidative stress variables.

Average values of F_2-isoprostanes, after log-transformation for approximate normality, were significantly higher in samples with non-zero values for apoA-I_{+16} compared with those with zero values (medians, 1576 compared with 1178 pmol/l; $P=0.02$); similarly for apoA-I_{+16} + apoA-I_{+32} (1542 compared with 1165 pmol/l; $P=0.003$). The comparison for apoA-I_{+32} was not significant ($P=0.14$), but this was probably due to the small number ($n=9$) of non-zeros, as F_2-isoprostanes were higher in the non-zeros than the zeros (medians, 1484 compared with 1226 pmol/l). Unexpectedly, F_2-isoprostanes were significantly positively related to apoA-II-1 ($r=0.09$, $P=0.03$) and negatively related, with virtually identical strength, to apoA-II-2 ($r=-0.10$, $P=0.02$). There was no evidence of an association between F_2-isoprostanes and apoA-II-3 ($P=0.89$).

There were few significant associations between coronary risk factors and the continuous biochemical markers (Table 3), whereas in only two situations was there any evidence (all other $P>0.1$) of a difference in associations between cases and unique controls. Both F_2-isoprostanes (positively) and apoA-II-1 (negatively) were associated with HDL, F_2-isoprostanes were significantly positively related to BMI (body mass index) and total serum cholesterol and, among controls only, apoA-II-2 was negatively associated with CRP.

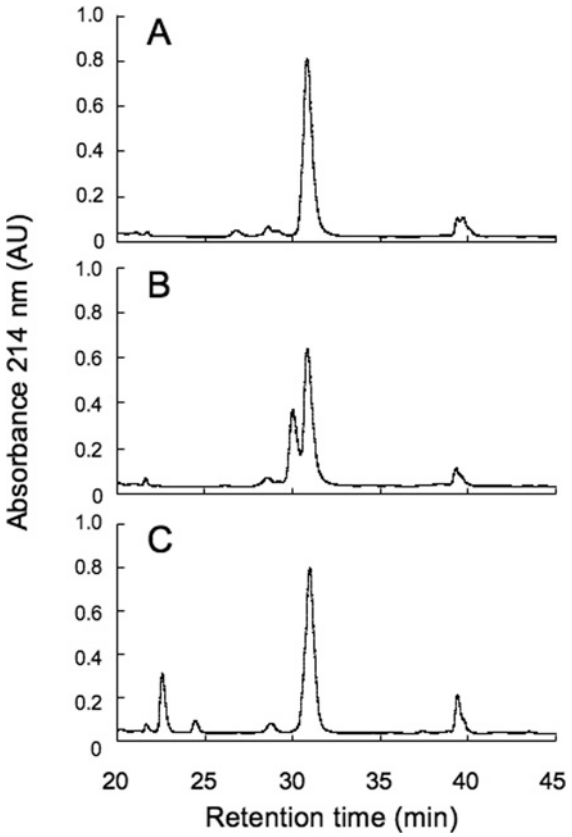

Figure 2 Typical HPLC traces of HDL samples isolated from plasma

(A) Sample containing no oxidized apoA-I; (B) sample containing apoA-I and apoA-I_{+16}; and (C) sample containing apoA-I and apoA-I_{+32}. AU, arbitrary units.

Table 2 Summary measures for unique controls and cases

Age and gender were matched between controls and cases. SBP, systolic blood pressure.

(a) Continuous variables

	Controls				Cases			
Variable	*n*	Minimum	Median	Maximum	*n*	Minimum	Median	Maximum
Age (years)	420	19	57	85	227	20	58	85
SBP (mmHg)	420	91	132	200	227	82	135	198
Total cholesterol (mmol/l)	419	3.1	5.5	8.9	226	2.9	5.9	11.5
HDL-cholesterol (mmol/l)	409	0.47	0.94	2.31	211	0.41	0.83	2.33
Triacylglycerols (mmol/l)	409	0.30	1.49	6.48	211	0.39	1.68	18.18
BMI (kg/m^2)	420	16.8	26.0	45.9	226	17.0	26.7	44.1
Fibrinogen (g/l)	411	1.56	3.20	5.10	223	1.37	3.45	7.19
IL-6 (pg/ml)	372	0.12	1.29	20.00	204	0.25	1.78	20.00
CRP (mg/l)	360	0.16	1.10	39.00	200	0.16	1.87	123.00
F_2-Isoprostanes (pmol/l)	417	255	1250	65 287	227	332	1146	49 666
Apo-II-1 (%)	334	0.0	33.6	100.0	205	0.0	34.7	100.0
Apo-II-2 (%)	334	0.0	60.9	84.9	205	0.0	62.0	96.8

(b) Categorical variables

	Control		Cases	
Variable	*n*	%	*n*	%
Male gender	325	77	175	77
Current smokers	74	18	55	24
Ex-smokers	169	40	105	46
ApoA-I_{+16} (positive)	67	20	49	24
ApoA-I_{+32} (positive)	7	2	2	1
ApoA-I_{+16} + apoA-I_{+32} (positive)	71	21	51	25
ApoA-II-3 (positive)	43	13	20	10

Table 3 Age- and gender-adjusted linear regressions between continuous markers of oxidative stress and coronary risk factors

Results shown are the slope of the fitted line (after transforming the dependent variable by log base e for F_2-isoprostanes, raising to the power 1/3 for apoA-II-1 and raising to the power 3 for apoA-II-2), the *P* value for the adjusted significance of the risk factor and R^2, and the percentage of variation in the dependent variable explained by the regression. All of the results are for all participants taken together, except where there was a significant interaction with control/case status (apoA-II-1 and HDL-cholesterol, and apoA-II-2 and CRP). SBP, systolic blood pressure.

	Dependent variable								
	F_2-Isoprostanes			ApoA-II-1			ApoA-II-2		
Variable	Slope	*P* value	Variation	Slope	*P* value	Variation	Slope	*P* value	Variation
SBP	− 0.0019	0.23	19 %	− 0.0002	0.87	2.2 %	201	0.56	2.2 %
Total cholesterol	0.0666	0.01	20 %	− 0.0198	0.39	2.4 %	4450	0.43	2.3 %
HDL-cholesterol	0.4389	< 0.0001	21 %	− 0.2791/− 0.6735	0.01/< 0.0001	3.1 %/13 %	29 541	0.19	2.5 %
Triacylglycerols	0.0075	0.73	18 %	− 0.0092	0.63	2.2 %	2543	0.59	2.3 %
BMI	0.0240	0.002	21 %	0.0067	0.32	2.4 %	− 686	0.68	2.2 %
Fibrinogen	0.0330	0.44	20 %	0.0552	0.13	3.0 %	− 1032	0.91	2.3 %
IL-6	0.0116	0.25	19 %	0.0118	0.18	2.6 %	− 473	0.83	2.3 %
CRP	− 0.0016	0.71	20 %	0.0028	0.46	2.8 %	− 6786/562	0.01/0.60	3.7 %/2.8 %

Table 4 ORs (95% CIs) for CHD

*Without CHD at baseline; †matched for age and gender; ‡adjusted for smoking status (current/ex-/never), SBP (systolic blood pressure), BMI, total cholesterol, HDL-cholesterol, triacylglycerols, fibrinogen, IL-6 and CRP, as well as for age and gender by matching; §for an increase in one inter-quartile range in unique controls (1013 pmol/l); ||for an increase in ten percentage points. + ve vs − ve, positive compared with negative.

	All subjects		Subjects free of CHD*	
Variable	Unadjusted†	Adjusted‡	Unadjusted†	Adjusted‡
F_2-Isoprostanes (pmol/l)				
First third (254–954)	1	1	1	1
Second third (955–1538)	1.31 (0.87–1.97)	1.35 (0.73–2.52)	1.41 (0.88–2.26)	1.36 (0.66–2.76)
Third third (1539–65288)	1.04 (0.64–1.71)	0.88 (0.40–1.95)	1.16 (0.67–2.00)	0.93 (0.39–2.24)
Continuous§	1.02 (0.98–1.06)	1.04 (0.93–1.15)	1.02 (0.98–1.06)	1.04 (0.93–1.16)
ApoA-I_{+16} (+ ve vs − ve)	1.24 (0.81–1.89)	1.08 (0.57–2.02)	1.31 (0.83–2.08)	1.15 (0.57–2.34)
ApoA-I_{+32} (+ ve vs − ve)	0.57 (0.12–2.75)	0.41 (0.03–6.16)	0.67 (0.14–3.30)	0.32 (0.01–7.36)
ApoA-I_{+16} + apoA-I_{+32} (+ ve vs − ve)	1.24 (0.81–1.89)	1.05 (0.56–1.95)	1.30 (0.82–2.06)	1.10 (0.55–2.22)
ApoA-II-3 (+ ve vs − ve)	0.68 (0.38–1.20)	0.76 (0.31–1.84)	0.57 (0.29–1.10)	0.51 (0.18–1.44)
ApoA-II-1 (%)				
First third (0–29.13)	1	1	1	1
Second third (29.14–40.10)	1.28 (0.83–1.98)	1.09 (0.58–2.05)	1.38 (0.85–2.24)	0.98 (0.49–1.94)
Third third (40.11–100)	1.42 (0.93–2.17)	1.12 (0.58–2.18)	1.46 (0.91–2.37)	1.11 (0.50–2.46)
Continuous||	1.09 (1.01–1.17)	1.03 (0.91–1.15)	1.09 (1.00–1.19)	1.01 (0.88–1.16)
ApoA-II-2 (%)				
First third (0–52.10)	1	1	1	1
Second third (52.11–67.23)	1.10 (0.72–1.68)	1.32 (0.72–2.42)	1.20 (0.76–1.91)	1.55 (0.77–3.11)
Third third (67.24–96.79)	0.95 (0.61–1.48)	1.21 (0.66–2.20)	1.05 (0.64–1.73)	1.39 (0.68–2.84)
Continuous||	0.96 (0.89–1.03)	1.02 (0.91–1.14)	0.98 (0.90–1.07)	1.06 (0.92–1.21)

The only significant coronary OR was for apoA-II-1 (Table 4): the odds of CHD rose by 9% [95% CI (confidence interval), 1–17%] for every extra 10% points. This significance was lost after adjustment for other coronary risk factors; indeed, it became non-significant ($P = 0.08$) after adjustment for fibrinogen alone.

At baseline, 19% of the cases, but none of the controls, had already experienced a heart attack. Removing these 43 cases, and their matched controls, made no appreciable changes to the ORs, with or without adjustments for potential confounding factors (Table 4).

DISCUSSION

In the present prospective study, we show the expected relationships between CHD and several established coronary risk factors, including BMI, cholesterol, CRP, HDL and smoking. However, we were unable to demonstrate any association between CHD and plasma markers of lipid or protein oxidation, namely F_2-isoprostanes and HDL-associated selectively oxidized apoA-I and apoA-II respectively. This is the first study examining the relationship between markers of oxidative damage and CHD outcome in a prospective study in a general population. We find no evidence that the parameters used to assess oxidative stress predict CHD, which is not consistent with the commonly held notion that oxidative stress significantly contributes to CHD outcome.

F_2-Isoprostanes are considered the 'gold standard' marker of oxidative stress [21]. The plasma levels of F_2-isoprostanes in the present study were similar to plasma isoprostanes measured in freshly collected plasma from previous human studies [22], indicating that samples were well preserved during storage and were free from artifactually high levels of lipid peroxidation products. A previous matched case-control study suggested that urinary F_2-isoprostanes may be a risk marker for CHD [23]; however, samples were collected after the development of disease so that it is impossible from that study to determine if F_2-isoprostanes are a predictor, or a result, of CHD. In a cohort of healthy young adults, coronary artery calcium was more likely in subjects with high rather than low plasma F_2-isoprostanes [24]; however, only 10% of the cohort had detectable disease, and our present findings do not support the proposal that plasma F_2-isoprostanes are predictive of future severe CHD. Finally, in a study of 298 healthy adults, gender was the strongest predictor of plasma F_2-isoprostanes, followed by smoking and CRP [25]. In our present study, cases and controls were matched for age and gender, and we observed no significant relationship with CRP.

The relative and independent contribution of coronary risk factors to systemic oxidative stress in a general

population has been assessed previously [26]. Smoking, diabetes and BMI were highly associated with oxidative stress, determined by urinary 8-epi-prostaglandin $F_{2\alpha}$. In agreement with that study, we also observed a positive relationship between BMI and F_2-isoprostanes in the present study. Interestingly, we observed a strong positive association between plasma F_2-isoprostanes and HDL, in keeping with the positive correlation between plasma F_2-isoprostanes and HDL-cholesterol as well as oxidized HDL apolipoproteins. It is also consistent with the finding that, in healthy subjects, the majority of lipid hydroperoxides [2] and F_2-isoprostanes (K. D. Croft and T. A. Mori, unpublished work) reside in HDL.

Importantly, the relationship, if any, between F_2-isoprostanes and protein oxidation is not known, yet protein oxidation could be important because it occurs in atherogenesis [27], and there is mounting evidence that HDL oxidation contributes to disease outcome [10,28]. Therefore, in the present study, we also employed a measure of protein oxidation that reflects HDL lipid oxidation independent of the oxidant species involved [12,13]. The latter is an important consideration as the oxidant(s) responsible for protein oxidation in human atherosclerosis remain poorly characterized [29]; however, the present results showed that, similar to plasma F_2-isoprostanes, the type of protein oxidation assessed does not predict CHD. Additional studies are required to determine whether this extends to other markers of HDL protein oxidation, such as chlorinated and/or nitrated species of apoA-I, of which several different types have been characterized and reported to be present in atherosclerotic lesions or circulation [30,31].

Unfortunately, a 'gold standard' for *in vivo* protein oxidation is presently not available. The method used in the present study is complex, although *in vitro* artifacts occurring during HDL isolation are not likely to explain the absence of the association between CHD and oxidized HDL proteins. This is because we observed good internal correlation between markers of plasma lipid and HDL protein oxidation: plasma F_2-isoprostanes were significantly higher in samples that contained oxidized HDL proteins, whereas such a correlation would not be expected if substantial oxidation of HDL occurred during its isolation. In addition, the samples used in the present study were stored in the presence of an antioxidant that associates with and prevents HDL lipid oxidation and hence formation of apoA-I_{+16} and apoA-I_{+32} [12,13]. In addition, the pattern of oxidized forms of apoA-I detected in the study samples differed greatly from that seen with *in-vitro*-oxidized HDL. Specifically, *in-vitro*-oxidized HDL apoA-I_{+32} always co-exists with apoA-I_{+16} independent of whether apolipoprotein oxidation is induced indirectly via lipid hydroperoxides (compare Figure 1B with Figure 2) or directly via a two-electron oxidant. Therefore our findings suggest, for the first time, that apoA-I_{+16} and apoA-I_{+32} are potential useful general markers for *in vivo* protein oxidation.

An unexpected finding of the present study was that increases in apoA-II-1 significantly elevated the risk of CHD. Little is known about the function of apoA-II, although *in vitro* apoA-II can affect hepatic lipase, LCAT (lecithin:cholesterol acyltransferase) and CETP, and there is evidence suggesting an association between apoA-II and insulin hypersensitivity [32]. Even less is known about the potential role of apoA-II variants, although it is increasingly appreciated that circulating apoA-II exists in different forms. Our present study is the first to document a potential negative association between CHD and circulating apoA-II without its C-terminal glutamine residue. We have no explanation for this putative association. In its simplest interpretation, it may suggest a relationship between CHD and carboxypeptidases. The inverse relationship between CHD and apoA-II-I disappeared after controlling for established coronary risk factors, and even after controlling for fibrinogen alone. This indicates that apoA-II-I may not be an independent risk factor for CHD and that there could be a relationship between apoA-II-I and thrombosis.

An important drawback of the present study is the low power to detect significance for the level of differences we have observed. This is manifested by the wide confidence limits shown in Table 4, particularly for the dichotomous measures. For these, largely because of the low proportion of non-zero values observed, we estimate that we would have required a sample of over 1 million subjects to have found the observed ORs significantly different from zero [18]. Even for the continuous measures, the comparisons between the thirds have low power: the highest observed OR had only approx. 40% power of being found significant at the standard 5% significance level. Given this, and the degree of inter-assay variation, which approached 10% for all our markers, further studies are required in this area, culminating in a meta-analysis to which the present study should contribute.

ACKNOWLEDGEMENTS

Fibrinogen, CRP and IL-6 assays were performed by Professor Gordon Lowe and Dr Ann Rumley (Department of Medicine, University of Glasgow, Glasgow, Scotland, U.K.).

FUNDING

This work was funded by the National Institutes of Health [grant number R01 HL73262]; and the National Health and Medical Research Council [grant numbers 358395, 401106]. R.S. is a recipient of

fellowships from the National Health and Medical Research Council and the University of Sydney Medical Foundation.

REFERENCES

1 Barter, P. J., Nicholls, S., Rye, K. A., Anantharamaiah, G. M., Navab, M. and Fogelman, A. M. (2004) Antiinflammatory properties of HDL. Circ. Res. **95**, 764–772
2 Bowry, V. W., Stanley, K. K. and Stocker, R. (1992) High density lipoprotein is the major carrier of lipid hydroperoxides in fasted human plasma. Proc. Natl. Acad. Sci. U.S.A. **89**, 10316–10320
3 Christison, J., Rye, K.-A. and Stocker, R. (1995) Exchange of oxidised cholesteryllinoleate between LDL and HDL mediated by cholesteryl ester transfer protein. J. Lipid Res. **36**, 2017–2026
4 Sattler, W., Christison, J. K. and Stocker, R. (1995) Cholesterylester hydroperoxide reducing activity associated with isolated high- and low-density lipoproteins. Free Radical Biol. Med. **18**, 421–429
5 Christison, J. K., Karjalainen, A., Brauman, J., Bygrave, F. and Stocker, R. (1996) Rapid reduction and removal of HDL- but not LDL-associated cholesteryl ester hydroperoxides by rat liver perfused *in situ*. Biochem. J. **314**, 739–742
6 Fluiter, K., Sattler, W., De Beer, M. C., Connell, P. M., van der Westhuyzen, D. R. and van Berkel, T. J. (1999) Scavenger receptor BI mediates the selective uptake of oxidized cholesterol esters by rat liver. J. Biol. Chem. **274**, 8893–8899
7 Niu, X., Zammit, V., Upston, J. M., Dean, R. T. and Stocker, R. (1999) Co-existence of oxidized lipids and α-tocopherol in all lipoprotein fractions isolated from advanced human atherosclerotic plaques. Arterioscler. Thromb. Vasc. Biol. **19**, 1708–1718
8 Upston, J. M., Niu, X., Brown, A. J. et al. (2002) Disease stage-dependent accumulation of lipid and protein oxidation products in human atherosclerosis. Am. J. Pathol. **160**, 701–710
9 Panzenböck, U. and Stocker, R. (2005) Formation of methionine sulfoxide-containing specific forms of oxidized high-density lipoproteins. Biochim. Biophys. Acta **1703**, 171–181
10 Pennathur, S., Bergt, C., Shao, B. et al. (2004) Human atherosclerotic intima and blood of patients with established coronary artery disease contain high density lipoprotein damaged by reactive nitrogen species. J. Biol. Chem. **279**, 42977–42983
11 Bergt, C., Pennathur, S., Fu, X. et al. (2004) The myeloperoxidase product hypochlorous acid oxidizes HDL in the human artery wall and impairs ABCA1-dependent cholesterol transport. Proc. Natl. Acad. Sci. U.S.A. **101**, 13032–13037
12 Garner, B., Witting, P. K., Waldeck, A. R., Christison, J. K., Raftery, M. and Stocker, R. (1998) Oxidation of high density lipoproteins. I. Formation of methionine sulfoxide in apolipoproteins AI and AII is an early event that correlates with lipid peroxidation and can be enhanced by α-tocopherol. J. Biol. Chem. **273**, 6080–6087
13 Garner, B., Waldeck, A. R., Witting, P. K., Rye, K.-A. and Stocker, R. (1998) Oxidation of high density lipoproteins. II. Evidence for direct reduction of HDL lipid hydroperoxides by methionine residues of apolipoproteins AI and AII. J. Biol. Chem. **273**, 6088–6095
14 Pankhurst, G., Wang, X. L., Wilcken, D. E. et al. (2003) Characterization of specifically oxidized apolipoproteins in mildly oxidized high density lipoprotein. J. Lipid Res. **44**, 349–355
15 Morrow, J. D. (2005) Quantification of isoprostanes as indices of oxidant stress and the risk of atherosclerosis in humans. Arterioscl. Thromb. Vasc. Biol. **25**, 279–286
16 MacMahon, S., Norton, R., Jackson, R. et al. (1995) Fletcher Challenge-University of Auckland Heart & Health Study: design and baseline findings. N.Z. Med. J. **108**, 499–502
17 Woodward, M., Rumley, A., Welsh, P., MacMahon, S. and Lowe, G. (2007) A comparison of the associations between seven haemostatic or inflammatory variables and coronary heart disease. J. Thromb. Haemostasis **5**, 1795–1800
18 Woodward, M. (2005) Epidemiology: Study Design and Data Analysis, 2nd., Chapman and Hall/CRC Press, Boca Raton
19 Sattler, W., Mohr, D. and Stocker, R. (1994) Rapid isolation of lipoproteins and assessment of their peroxidation by HPLC postcolumn chemiluminescence. Methods Enzymol. **233**, 469–489
20 Mori, T. A., Croft, K. D., Puddey, I. B. and Beilin, L. J. (1999) An improved method for the measurement of urinary and plasma F_2-isoprostanes using gas chromatography-mass spectrometry. Anal. Biochem. **268**, 117–125
21 Morrow, J. D. and Roberts, L. J. (1997) The isoprostanes: unique bioactive products of lipid peroxidation. Prog. Lipid Res. **36**, 1–21
22 Ward, N. C., Hodgson, J. M., Puddey, I. B., Mori, T. A., Beilin, L. J. and Croft, K. D. (2004) Oxidative stress in human hypertension: association with antihypertensive treatment, gender, nutrition and lifestyle. Free Radical Biol. Med. **36**, 226–232
23 Schwedhelm, E., Bartling, A., Lenzen, H. et al. (2004) Urinary 8-iso-prostaglandin F_{2a} as a risk marker in patients with coronary heart disease: a matched case-control study. Circulation **109**, 843–848
24 Gross, M., Steffes, M., Jacobs, Jr, D. R. et al. (2005) Plasma F_2-isoprostanes and coronary artery calcification: the CARDIA Study. Clin. Chem. **51**, 125–131
25 Block, G., Dietrich, M., Norkus, E. P. et al. (2002) Factors associated with oxidative stress in human populations. Am. J. Epidemiol. **156**, 274–285
26 Keaney, Jr, J. F., Larson, M. G., Vasan, R. S. et al. (2003) Obesity and systemic oxidative stress: clinical correlates of oxidative stress in the Framingham Study. Arterioscler. Thromb. Vasc. Biol. **23**, 434–439
27 Dean, R. T., Fu, S., Stocker, R. and Davies, M. J. (1997) Biochemistry and pathology of radical-mediated protein oxidation. Biochem. J. **324**, 1–18
28 Shishehbor, M. H., Aviles, R. J., Brennan, M. L. et al. (2003) Association of nitrotyrosine levels with cardiovascular disease and modulation by statin therapy. JAMA, J. Am. Med. Assoc. **289**, 1675–1680
29 Stocker, R. and Keaney, Jr, J. F. (2004) Role of oxidative modifications in atherosclerosis. Physiol. Rev. **84**, 1381–1478
30 Shao, B., Oda, M. N., Vaisar, T., Oram, J. F. and Heinecke, J. W. (2006) Pathways for oxidation of high-density lipoprotein in human cardiovascular disease. Curr. Opin. Mol. Ther. **8**, 198–205
31 Nicholls, S. J., Zheng, L. and Hazen, S. L. (2005) Formation of dysfunctional high-density lipoprotein by myeloperoxidase. Trends Cardiovasc. Med. **15**, 212–219
32 Weng, W. and Breslow, J. L. (1996) Dramatically decreased high density lipoprotein cholesterol, increased remnant clearance, and insulin hypersensitivity in apolipoprotein A-II knockout mice suggest a complex role for apolipoprotein A-II in atherosclerosis susceptibility. Proc. Natl. Acad. Sci. U.S.A. **93**, 14788–14794

Received 16 November 2007/12 May 2008; accepted 28 May 2008
Published as Immediate Publication 28 May 2008, doi:10.1042/CS20070404