High-density lipoprotein composition and paraoxonase activity in Type I diabetes

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ABSTRACT

Type I diabetes is associated with a high incidence of coronary heart disease (CHD), despite a normal or even increased concentration of high-density lipoprotein (HDL) cholesterol. This paradox may be explained by changes in the antioxidant capacity of HDL, related to paraoxonase (PON1) activity. HDL compositional changes in subjects with Type I diabetes may result in changes in PON1 activity that are associated with a higher incidence of CHD. Single-vertical-spin density-gradient ultracentrifugation was used to isolate seven HDL fractions from serum according to density. PON1 activity was measured in serum and in the HDL fractions using phenyl acetate as substrate. The mean recovery of PON1 activity in the HDL fractions was 87% (S.D. 12%). CHD risk was assessed using B-mode ultrasound to measure carotid artery intima-media thickness (IMT). Groups of 35 subjects with Type I diabetes (duration of diabetes 18 years (12–32 years) [median (interquartile range)]; glycated haemoglobin 7.67% (1.17%) and 24 non-diabetic control subjects were studied. Carotid IMT was greater in the diabetic subjects [0.60 (0.55–0.70) compared with 0.55 (0.45–0.64) mm; P = 0.042] and HDL cholesterol concentration was higher [1.53 (0.36) compared with 1.32 (0.34) mmol/l; P = 0.031]. There were qualitative differences in HDL in subjects with Type I diabetes: HDL particles were triacylglycerol-deplete, and there were greater numbers of the larger, more buoyant HDL particles. These properties were not those found to determine PON1 activity. PON1 activity increased as HDL particle density increased and particle size decreased; the increase in PON1 activity was associated with an increase in the ratio of the two HDL surface lipid components, phospholipid and unesterified cholesterol, as particle density increased. PON1 activity was similar in diabetic and non-diabetic subjects [121 (28) and 120 (36) μmol·min⁻¹·ml⁻¹ respectively; P = 0.887]. PON1 activity was not associated with carotid IMT in either group. Our results suggest that the PON1 activities of HDL particles relate to the density, size and composition of the particles. However, PON1 activity does not appear to contribute to the greater risk of CHD in subjects with Type I diabetes.

INTRODUCTION

A low concentration of high-density lipoprotein (HDL) cholesterol is a powerful and independent predictor of premature coronary heart disease (CHD) [1,2]. Type I diabetes, however, is associated with an increased incidence of CHD [3], despite normal or increased HDL cholesterol concentrations [4]. This paradox may be

Key words: carotid artery, coronary heart disease, HDL, intima-media thickness, lipoprotein composition, PON1, single-vertical-spin density-gradient ultracentrifugation.

Abbreviations: CHD, coronary heart disease; CV, coefficient of variation; HbA₁c, glycated haemoglobin; HDL, high-density lipoprotein; IMT, intima-media thickness; LDL, low-density lipoprotein; PON1, paraoxonase.

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explained by qualitative changes in HDL that affect its functional properties, such as its antioxidant capacity, related to paraoxonase (PON1) activity.

Klimov et al. [5] were the first to report a protective effect of HDL against iron-induced oxidation, while Parthasarathy et al. [6] demonstrated that total HDL isolated from normolipidaemic donors was able to greatly reduce the degradation of oxidized low-density lipoprotein (LDL) by macrophages. Mackness et al. [7] subsequently provided evidence that HDL could inhibit the formation of lipid peroxides in copper-incubated LDL by up to 90%, and that PON1, purified from HDL, prevented the formation of both thiobarbituric acid-reactive substances (mostly malondialdehyde) and lipo-peroxide [8]. Further data from the same laboratory showed that the copper-chelating action of PON1 was insufficient to explain the observed results, and therefore an enzymic process was proposed [9].

PON1 is isolated with HDL on ultracentrifugation of human serum [10]; separation of apolipoprotein A1 from PON1 during purification has proved difficult, suggesting that apolipoprotein A1 and PON1 are closely associated. An unusual property of PON1 is that the mature protein retains its hydrophobic N-terminal signal sequence. By expressing a mutant PON1 with a cleavable N-terminus in vitro, it has been demonstrated that PON1 associates with HDL through its N-terminus by binding phospholipids directly, rather than binding apolipoprotein A1 [11].

PON1 activity has been shown to be lower in subjects with Type I diabetes than in non-diabetic subjects. Reduced PON1 activity was demonstrated in 38 subjects with Type I diabetes when compared with 79 non-diabetic control subjects [12], and decreased PON1 specific activity was demonstrated in 78 subjects with Type I diabetes compared with 82 non-diabetic control subjects [13]. A reduced PON1 concentration, without changes in specific activity, was demonstrated in 168 subjects with Type I diabetes compared with 128 non-diabetic first-degree relatives [14]. Reduced PON1 activity in Type I diabetes is unrelated to differences in PON1 genotype distribution [13,14]. The lowered PON1 activity may therefore be related to decreased specific activity of PON1 (possibly due to glycation of the enzyme or to the presence of a circulating inhibitor) or to a decreased serum concentration. In vitro incubation of human HDL in the presence of 25 mmol/l glucose has been shown to result in a decrease in PON1 activity [15], suggesting the possibility that glycation of the enzyme affects specific activity in diabetes. It is also possible that disturbances in the interaction of PON1 with HDL in Type I diabetes result in decreased activity, associated either with decreased specific activity or decreased mass of PON1. We hypothesized that HDL compositional changes in Type I diabetes are associated with reductions in PON1 activity, and that PON1 activity is associated with carotid artery intimamedia thickness (IMT), a surrogate marker for CHD [16].

PON1 exhibits a genetic polymorphism due to an amino acid substitution at position 192 (a glutamine to arginine substitution) of the PON1 gene coding region. The B-alloenzyme (arginine at position 192) hydrolyses paraoxon at a higher rate than the A-alloenzyme (glutamine at position 192). Although the genetic polymorphism can therefore be demonstrated phenotypically when using paraoxon as substrate, the alloenzymes have similar activities towards other substrates, such as phenyl acetate. The role of the PON1 polymorphism in CHD risk is controversial, and the physiological substrate for PON1 is presently unknown. We therefore elected to measure PON1 activity in serum and in separated HDL fractions from subjects with Type I diabetes and from non-diabetic control subjects using phenyl acetate as the substrate for the assay. Genotypic variation at position 192 would not therefore confound differences in PON1 activity between groups. This seemed a reasonable approach, as no differences in genotypic frequencies between subjects with and without Type I diabetes have been demonstrated [13,14]. Activity measured using phenyl acetate as substrate has been thought to more closely reflect PON1 concentration.

MATERIALS AND METHODS

Subjects

Subjects with Type I diabetes were recruited consecutively from the diabetic clinics at St Mary’s and St Charles Hospitals (London, U.K.); subjects had required insulin treatment within 1 year of diagnosis of diabetes, and had undetectable fasting C-peptide concentrations (<100 pmol/l). Subjects were free from other significant medical conditions and had creatinine concentrations <140 μmol/l. Healthy control subjects were recruited from hospital staff. All subjects attended following a 12 h overnight fast for venesection and for clinical assessment by the same investigator (J.V.). Diabetic subjects omitted their morning insulin injection on the morning of the study until after venesection was performed. The presence of retinopathy was defined as the detection of at least one microaneurism by direct ophthalmoscopy. The presence of peripheral neuropathy was defined as the absence of both ankle jerks and/or the bilateral absence of vibration sensation at the medial maleolus using a 128 Hz tuning fork. The presence of peripheral vascular disease was defined as the absence to palpation of all four foot pulses or a previous digital (or more proximal limb) anamputation performed for ischaemia. Blood pressure was recorded while subjects rested in a sitting position, using a mercury column sphygmomanometer with appropriate cuff size. A single early-morning urine sample was collected.
collected for measurement of the albumin/creatinine ratio; a ratio of > 2.0 was taken to indicate the presence of microalbuminuria. Local Ethical Committee approval was granted, and informed consent was obtained from all participating subjects.

Measurement of carotid artery IMT
CHD risk was assessed in each subject by measuring carotid artery IMT. All examinations were performed using an ATL 3000 HDI system (high-definition imaging; Advanced Technology Laboratories, Seattle, WA, U.S.A.). A 7–4 MHz linear-array transducer with broadband technology was used to scan all subjects. Ultrasound parameters (post-processing map, dynamic range, persistence, power output and transmit gain control) were pre-set and kept constant throughout the study. Magnification and depth could be adjusted depending upon patient anatomy and size.

Regular use of an RMI (model 415; Gammax Radiation Measuring Instruments Ltd, Nottingham, U.K.) test object ensured system accuracy in sensitivity, distance measurements, and axial and lateral resolution measurements. A single experienced ultrasonographer performed all measurements, and images were stored on magneto-optical discs for analyses.

Subjects were examined in the supine position. All measurements were taken in longitudinal planes. The IMT was measured on B-mode image, 2.0 cm proximal to the carotid bifurcation on the posterior wall of the artery. IMT was defined as the distance on the posterior wall between the leading edge of the I band and the leading edge of the M band [17]. IMT measurements of left and right carotid arteries were recorded, and carotid artery IMT was calculated as the mean of the left and right carotid artery measurements. The reproducibility of measurements of IMT has been assessed in The Irvine Laboratory by Veller et al. previously [17]; the coefficient of variation (CV) for the measurement of IMT was 5.7%.

Isolation of HDL
Single-vertical-spin density-gradient ultracentrifugation was used to isolate seven HDL fractions from serum according to density [18]. A Beckman Vti 90 vertical rotor, an LK100 Beckman ultracentrifuge and 5 ml Optiseal polyallomer centrifuge tubes (Beckman Instruments, Inc.) were used. Serum was density-adjusted to 1.31 g/ml with KBr, and then overlaid with 150 mM NaCl solution. Centrifugation was for 44 min at 462,000 g. In our hands, the CV for the distribution of density post-centrifugation was 0.3%. If HDL prepared previously by sequential ultracentrifugation was density-adjusted to 1.31 g/ml, overlaid with 150 mM NaCl and then subjected to single-vertical-spin density-gradient ultracentrifugation, recovery in the seven fractions of cholesterol associated with the HDL was 100%. HDL fractions separated from three different sera underwent gradient gel electrophoresis to establish particle size; increasing HDL particle density corresponded to decreasing particle size (Figure 1).

Measurement of PON1 activity
PON1 activity was determined by measuring arylesterase activity in the presence of eserine, using phenyl acetate as substrate [19]. PON1 and the cholinesterases (acetylcholinesterase and pseudocholinesterase) are the enzymes responsible for the arylesterase activity of serum. Eserine inhibits arylesterase activity attributable to the cholinesterases.

The constituents of the reaction mixture were 20 mM Tris/HCl, pH 8.2, and 0.9 mM CaCl$_2$. Added to 2.0 ml of a 25 °C reaction mixture were 2.5 μl of serum (20 μl of serum diluted to 1:8 with reaction mixture) and 200 μl of eserine solution (final eserine concentration in the reaction mixture of 5 μM). This was incubated for 20 min, to allow the eserine to inhibit the arylesterase activity of the serum sample attributable to acetylcholinesterase and pseudocholinesterase. The reaction was then initiated by the addition of 300 μl of phenyl acetate solution (final phenyl acetate concentration in the reaction mixture of 1 mM), and the initial rates of hydrolysis of phenyl acetate were determined spectrophotometrically at 270 nm, using the molar absorption coefficient for phenyl acetate, which is 1310 M$^{-1}$ cm$^{-1}$. The intra-assay CV, based on 12 measurements performed on the same serum, was 1.7% at 129 μmol·min$^{-1}$·ml$^{-1}$ and 1.8% at 202 μmol·min$^{-1}$·ml$^{-1}$. The inter-assay CV, based on 12 measurements performed on the same serum, was 3.3% at 123 μmol·min$^{-1}$·ml$^{-1}$.

PON1 activity was also measured in the seven HDL fractions prepared by single-vertical-spin density-gradient ultracentrifugation. The arylesterase activity of PON1 is calcium-dependent. Albumin (to which the majority of calcium is bound in serum) and calcium
separate out with the highest-density HDL fractions during single-vertical-spin density-gradient ultracentrifugation. In order to maintain the arylesterase activity of PON1 in the lower-density HDL fractions following fraction separation, it was therefore necessary to add calcium-containing reaction mixture to the fractions immediately following separation. Samples of the HDL fractions were therefore diluted to 1:8 (v/v) with reaction mixture immediately after separation, and were then assayed for PON1 activity in the same way as for serum. HDL fractions were not therefore dialysed prior to the measurement of PON1 activity. We have demonstrated that the addition of KBr to serum at concentrations reached in the HDL fractions does not affect the measured PON1 activity of the serum (results not shown). The mean recovery of PON1 activity in the seven HDL fractions was 87% (S.D. 12%).

Analytical methods
Total cholesterol, unesterified cholesterol, triacylglycerol and phospholipid were measured enzymically (Alpha Laboratories Ltd, UK). Measurements were performed on serum using a Cobas-Mira analyser (Hoffmann-La Roche & Co., Diagnostics Division, Basle, Switzerland), according to the manufacturer’s protocols. Measurements were also performed on the HDL fractions following dialysis against a buffered solution. The concentration of esterified cholesterol was calculated as the difference between the concentrations of total cholesterol and unesterified cholesterol. Creatinine, glycated haemoglobin (HbA1c) and fasting glucose concentrations were measured using standard laboratory techniques. Apolipoprotein A1 and apolipoprotein B100 were measured in fractions and serum using rate immunonephelometry on a Beckman ARRAY analyser.

Statistical analyses
To compare a continuous variable between the two groups, Student’s unpaired t-test and the Mann–Whitney test were used for variables with normal and skewed distributions respectively. Continuous variables with normal distributions are expressed as mean (S.D.); continuous variables with skewed distributions are expressed as median (interquartile range). To compare categorical variables between the two groups, Fisher’s exact test was performed. Categorical variables are expressed as number of subjects with the percentage in each category.

Linear regression was used to assess the relationship between continuous variables. Residual analyses were performed: a scatterplot of the residuals versus the fitted values established homoscedasticity; the residuals were tested for normality using the Shapiro–Wilk W test. If the residuals did not have constant variance or were not normally distributed, then the variable was log e-transformed prior to regression.

Seven HDL fractions were isolated from the density gradient, in each of which seven different parameters were measured. Comparison of HDL fraction composition between the two groups could therefore result in 49 different comparisons. This would require tests of significance to be made with a Bonferroni correction; a two-tailed P-value of < 0.005 would be required to achieve significance. In a small study, this would result in a high risk of type 2 errors. Therefore multiple comparisons were avoided in the analysis of lipoprotein composition. Each parameter measured was assigned an area under the curve when the parameter was plotted against the seven HDL fractions of increasing density, and areas under the curve were compared between the two groups. Therefore, for statistical evaluations, a two-tailed P-value of < 0.05 was considered significant.

The Arcus Quickstat Biomedical package was used for the analyses (Longman Software Publishing, Cambridge, U.K.).

RESULTS

Subject characteristics
A total of 35 subjects with Type I diabetes and 24 non-diabetic control subjects were recruited. All underwent clinical evaluation, venesection and ultrasound examination. Table 1 shows the demographic, clinical and ultrasound characteristics of the two groups. Ages, sex distribution and body-mass index were similar in the two groups. Three subjects with Type I diabetes were known to have CHD (9%); one had undergone coronary artery bypass grafting, one had undergone single-vessel angioplasty, and one had stable exertional angina. Five diabetic subjects had peripheral vascular disease (14%); four had absent foot pulses and one had undergone digital amputations for ischaemia. One patient was known to have cerebrovascular disease (3%), having experienced a transient ischaemic attack. In addition, 19 subjects with Type I diabetes had retinopathy (54%) and eight had neuropathy (23%); 12 had microalbuminuria (34%), although none had frank proteinuria. Carotid IMT was significantly greater in the diabetic subjects [median (interquartile range) 0.60 (0.55–0.70) mm, compared with 0.55 (0.45–0.64) mm; P = 0.042], and this difference, when assessed in a multiple regression model, remained significant when differences in age and smoking status between the groups were taken into account. Carotid IMT was associated with age in both diabetic (r = 0.52; P = 0.0015) and non-diabetic (r = 0.47; P = 0.022) subjects, and with systolic blood pressure in the diabetic subjects (r = 0.62; P < 0.0001). Table 2 gives the biochemical characteristics of the two groups. HDL chol-
Table 1  Demographic, clinical and ultrasound characteristics of subjects with Type I diabetes and non-diabetic control subjects

Categorical variables are expressed as numbers in each category with percentages given in parentheses; continuous variables are expressed as mean (S.D.) if the variable has a normal distribution, or as median (interquartile range) if the variable has a skewed distribution.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Type I diabetic subjects</th>
<th>Control subjects</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>35</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Number of males (%)</td>
<td>22 (63)</td>
<td>15 (63)</td>
<td>1.000</td>
</tr>
<tr>
<td>Age (years)</td>
<td>40 (29–52)</td>
<td>34 (30–44)</td>
<td>0.269</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>24.6 (2.1)</td>
<td>24.9 (2.6)</td>
<td>0.732</td>
</tr>
<tr>
<td>Number of smokers (%)</td>
<td>10 (29)</td>
<td>2 (8)</td>
<td>0.098</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>130 (121–149)</td>
<td>121 (113–127)</td>
<td>0.004</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>78 (8)</td>
<td>78 (8)</td>
<td>0.934</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>18 (12–32)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Carotid IMT (mm)</td>
<td>0.60 (0.55–0.70)</td>
<td>0.55 (0.45–0.64)</td>
<td>0.042</td>
</tr>
</tbody>
</table>

Table 2  Biochemical characteristics of subjects with Type I diabetes and non-diabetic control subjects

Categorical variables are expressed as numbers in each category with percentages; continuous variables are expressed as mean (S.D.) if the variable has a normal distribution, or as median (interquartile range) if the variable has a skewed distribution.

<table>
<thead>
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<th>Parameter</th>
<th>Type I diabetic subjects</th>
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<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>35</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.67 (1.17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>10.2 (8.4–15.4)</td>
<td>4.9 (4.4–5.3)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Creatinine (µmol/l)</td>
<td>104 (13)</td>
<td>102 (13)</td>
<td>0.469</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.53 (0.36)</td>
<td>1.32 (0.34)</td>
<td>0.031</td>
</tr>
<tr>
<td>Serum apolipoprotein A1 (g/l)</td>
<td>1.59 (1.40–1.71)</td>
<td>1.47 (1.38–1.60)</td>
<td>0.084</td>
</tr>
<tr>
<td>Serum apolipoprotein B100 (g/l)</td>
<td>0.80 (0.74–1.01)</td>
<td>0.80 (0.69–1.06)</td>
<td>0.752</td>
</tr>
<tr>
<td>Serum total cholesterol (mmol/l)</td>
<td>5.07 (0.82)</td>
<td>4.96 (0.90)</td>
<td>0.615</td>
</tr>
<tr>
<td>Serum unesterified cholesterol (mmol/l)</td>
<td>1.29 (0.21)</td>
<td>1.25 (0.26)</td>
<td>0.540</td>
</tr>
<tr>
<td>Serum esterified cholesterol (mmol/l)</td>
<td>3.78 (0.61)</td>
<td>3.70 (0.66)</td>
<td>0.645</td>
</tr>
<tr>
<td>Serum triacylglycerol (mmol/l)</td>
<td>0.87 (0.68–1.12)</td>
<td>0.98 (0.71–1.49)</td>
<td>0.308</td>
</tr>
<tr>
<td>Serum phospholipid (mmol/l)</td>
<td>2.85 (0.39)</td>
<td>2.62 (0.40)</td>
<td>0.031</td>
</tr>
</tbody>
</table>

HDL composition

Concentrations of esterified cholesterol, unesterified cholesterol, triacylglycerol, phospholipid and apolipoprotein A1 in the fractions of the HDL density gradient were compared between the two groups. There were significant differences between the HDL fractions derived from subjects with Type I diabetes and those derived from control subjects, as determined by area-under-the-curve analyses of the composites (Figure 2). Diabetic subjects had significantly greater areas under the curve than control subjects for esterified cholesterol [median (interquartile range): 4.37 (3.78–5.31) and 3.98 (3.57–4.51) respectively; P = 0.038] (Figure 2a) and for phospholipid [4.65 (4.03–5.79) and 4.11 (3.58–4.30) respectively; P = 0.003] (Figure 2b). It can be seen from the graphs that the greater values for HDL esterified cholesterol and phospholipid in the subjects with Type I diabetes are attributable largely to increased concentrations of esterified cholesterol and phospholipid associated with the larger, more buoyant HDL fractions. The areas under the curve for unesterified cholesterol [1.11 (0.92–1.36) and 1.03 (0.79–1.16); P = 0.088] and for triacylglycerol [0.52 (0.43–0.68) and 0.58 (0.47–0.78); P = 0.071] were not significantly different in the Type I diabetic and non-diabetic subjects respectively. Although apolipoprotein A1 concentrations were higher in the larger, more buoyant HDL fractions of the subjects with Type I diabetes, the area under the curve for apolipoprotein A1 was not statistically significantly higher in the diabetic group [4.30 (4.02–5.24)] than in the control group [4.16 (3.73–4.47); P = 0.112] (Figure 2c).
Figure 2 Concentrations of esterified cholesterol (a), phospholipid (b) and apolipoprotein A1 (c) in HDL fractions of increasing density isolated from the serum of subjects with Type I diabetes (■) and non-diabetic control subjects (●).

For each of the two groups, mean concentrations in each fraction are plotted; error bars represent S.E.M. Areas under the curves between HDL fractions 1 and 7 were calculated for statistical comparisons between the two groups. The differences for esterified cholesterol ($P = 0.038$) and for phospholipid ($P = 0.003$) were statistically significant.

In order to establish whether the differences in HDL fraction lipid concentrations were due to greater lipid loading or increased numbers of HDL particles, the lipid concentrations were expressed as molar ratios relative to the apolipoprotein A1 concentration in each fraction. This can only approximate the lipid loading of each HDL particle, since the number of apolipoprotein A1 molecules per HDL particle can vary from four to two as HDL particle size decreases and density increases. To calculate the molar ratios, it was necessary to convert the measured concentrations of apolipoprotein A1 into mmol/l (the $M_r$ of apolipoprotein A1 is 28,000). The areas under the curve for the molar ratios in the HDL-containing fractions in the two groups were then compared. There were no significant differences between the two groups in the molar ratios of esterified cholesterol [median (interquartile range): diabetic subjects, 198 (174–235); control subjects, 201 (177–221); $P = 0.883$] and unesterified cholesterol [diabetic subjects, 53 (47–63); control subjects, 52 (44–60); $P = 0.594$] to apolipoprotein A1 in HDL-containing fractions. Therefore the significantly greater values for HDL esterified cholesterol in the subjects with Type I diabetes were likely to be attributable to increased numbers of the larger, more buoyant HDL particles, rather than to increased loading of each particle with esterified cholesterol. The molar ratios of triacylglycerol to apolipoprotein A1 were significantly lower in the subjects with Type I diabetes [27 (21–34)] than in control subjects [33 (26–47); $P = 0.039$] (Figure 3). The reduction in triacylglycerol per HDL particle in the subjects with Type I diabetes was seen in all the HDL fractions, regardless of HDL particle size and density. Despite a significant decrease in the triacylglycerol load per HDL particle, the decrease in total HDL triacylglycerol was not significant in the subjects with Type I diabetes. This can be attributed to the likely greater numbers of the larger, more buoyant HDL particles in the subjects with Type I diabetes, so that, although triacylglycerol load per HDL particle was reduced, total HDL triacylglycerol was not significantly altered. An increase in the molar
HDL composition and paraoxonase activity in Type I diabetes

Figure 4 Esterified cholesterol/triacylglycerol (a), phospholipid/unesterified cholesterol (b) and phospholipid/esterified cholesterol (c) concentration ratios in HDL fractions of increasing density isolated from serum of subjects with Type I diabetes (+) and non-diabetic control subjects (E). For each of the two groups, mean ratios in each fraction are plotted; error bars represent S.E.M. Areas under the curves between HDL fractions 1 and 7 were calculated for statistical comparisons between the two groups. The differences for the esterified cholesterol/triacylglycerol (triglyceride) concentration ratio were statistically significant (P < 0.006). Ratios of phospholipid to apolipoprotein A1 in the subjects with Type I diabetes failed to achieve statistical significance [diabetic subjects, 204 (187–235); control subjects, 192 (172–209); P = 0.070]. It is therefore likely that the increase in HDL phospholipid was attributable to the increase in the numbers of the larger, more buoyant HDL particles.

Differences in the ratios of the core and surface lipid components of HDL in Type I diabetes were assessed by area-under-the-curve analyses (illustrated graphically in Figure 4). The ratios of the core HDL lipid components, i.e. esterified cholesterol/triacylglycerol, were significantly greater in the subjects with Type I diabetes [9.55 (6.98–10.61)] than in control subjects [6.72 (4.91–8.60); P = 0.006] (Figure 4a). It is evident from Figure 4(a) that the ratio is greater in all of the HDL-containing fractions in the subjects with Type I diabetes, regardless of HDL particle size and density. The ratios of the surface HDL lipid components, i.e. phospholipid/unesterified cholesterol, were similar in diabetic [4.26 (3.87–4.92)] and control [4.08 (3.75–4.64); P = 0.440] subjects (Figure 4b). The ratios of the main HDL surface lipid component, phospholipid, to the main HDL core lipid component, esterified cholesterol, were also similar in Type I diabetic and control subjects [1.05 (0.98–1.13) and 0.99 (0.93–1.12) respectively; P = 0.125] (Figure 4c).

PON1 activity

PON1 activity was measured in serum and in the HDL fractions. There was no significant difference in serum PON1 activity between the two groups [diabetic group, 121 (28) μmol·min⁻¹·ml⁻¹; control group, 120 (36) μmol·min⁻¹·ml⁻¹; mean (S.D.); P = 0.887]. Neither were there any significant differences in the PON1 activities of the HDL fractions between the two groups, assessed by area under the curve analysis [diabetic group, 321 (70); control group, 321 (86); P = 0.995] (Figure 5a). In an attempt to estimate the PON1 activity per HDL particle, PON1 activity was expressed in each fraction as the molar ratio of apolipoprotein A1 concentration (mmol of phenyl acetate hydrolysed·min⁻¹·mmol⁻¹ apolipoprotein A1). There was no significant difference in the molar ratios of PON1 activity to apolipoprotein A1 concentration between the two groups, as assessed by area under the curve analysis [diabetic group, 12.0 × 10⁻³ [(10.3–13.8) × 10⁻³]; control group, 12.9 × 10⁻³ [(11.3–16.0) × 10⁻³]; P = 0.272] (Figure 5b).

It was apparent, however, that the molar ratios of PON1 activity to apolipoprotein A1 concentration (the estimate of PON1 activity per particle) increased progressively as HDL particle density increased (and, correspondingly, as particle size decreased). The molar ratios were greatest in the smallest, most dense HDL particles from both groups (Figure 5b).

HDL compositional determinants of PON1 activity

There were two lipid compositional changes that paralleled the change in the molar ratios of PON1 activity to apolipoprotein A1 concentration as HDL particle size
decreased and particle density increased. First, the ratio of the two HDL surface lipid components, i.e. phospholipid/unesterified cholesterol, increased as HDL particle size decreased and particle density increased (Figure 4b). Secondly, the ratio of the major HDL surface lipid component, phospholipid, to the major HDL core lipid component, esterified cholesterol, increased as HDL particle size decreased and particle density increased (Figure 4c).

**PON1 activity and carotid IMT**

There were no significant correlations between serum PON1 activity and carotid IMT (Figure 6a) in the diabetic group, in the control group or in both groups analysed together. PON1 activity was associated with HDL cholesterol concentration in both diabetic ($r = 0.39; P = 0.0204$) and non-diabetic ($r = 0.58; P = 0.0027$) subjects; there was no difference in the slope of the association according to diabetic status ($P = 0.23$ for the interactive term) (Figure 6b). PON1 activity was associated with apolipoprotein A1 concentration in the non-diabetic subjects ($r = 0.53; P = 0.0081$), but the association failed
to achieve statistical significance in the diabetic subjects ($r = 0.18; P = 0.31$) (Figure 6c).

**DISCUSSION**

Although we have demonstrated significant HDL compositional changes in the subjects with Type I diabetes studied, as well as a greater carotid IMT, we could not demonstrate either reduced PON1 activity in the subjects with Type I diabetes or an association of PON1 activity with carotid IMT.

Three studies have reported low PON1 activity in subjects with Type I diabetes. Mackness et al. [12] described reduced PON1 activity in 38 subjects with Type I diabetes compared with 79 non-diabetic control subjects. Although glycaemic control was good, the subjects with Type I diabetes were older than in the present study [51 (S.D. 12) years], were dyslipidaemic (mean total cholesterol 7.29 mmol/l; geometric mean fasting triacylglycerol 1.97 mmol/l), and a third were known to have CHD. The same group described reduced PON1 activity in 78 subjects with Type I diabetes compared with 82 non-diabetic control subjects [13].

Again, the subjects with Type I diabetes were older than in the present study [47.3 (12.4) years] and were dyslipidaemic (mean total cholesterol 6.20 mmol/l; geometric mean fasting triacylglycerol 1.57 mmol/l), and almost half of the subjects had neuropathy. Furthermore, glycaemic control in the 78 subjects with Type I diabetes was poor [HbA$_1c$ 11.4% (4.0%)], so that glycation of PON1 may have contributed significantly to the conclusion of reduced specific PON1 activity [15]. The subjects with Type I diabetes in the present study had good glycaemic control, and, as described in other studies of subjects with Type I diabetes with good glycaemic control and without overt proteinuria, had normal lipid profiles. Boemi et al. [14] described a reduced PON1 concentration, paralleled by a decrease in activity measured using paraoxon as substrate, in 168 subjects with Type I diabetes compared with 128 non-diabetic first-degree relatives. While age distribution and lipid parameters were similar to those in the present study, glycaemic control was worse [HbA$_1c$ 9.3% (1.8%)]. Differences in subject characteristics compared with previous studies could therefore account for the normal PON1 activity in the subjects with Type I diabetes in the present study. It could be argued that the number of subjects involved in the present study was too small to be able to reach unambiguous conclusions concerning relative PON1 activities in Type I diabetic and non-diabetic subjects. However, a post hoc power calculation suggested that the present study was adequately powered to demonstrate differences in PON1 activity between the groups. The S.D. of PON1 activity measured using our assay on a log scale was 0.26. Mackness et al. [12] previously observed a difference of this magnitude between a group of subjects with Type I diabetes and a non-diabetic group. Therefore, with 90% power and 5% significance, we would have been able to detect such a difference with approximately 21 subjects in each group.

Carotid IMT has proved to be a reliable surrogate marker for CHD [16]. Other studies have demonstrated a greater common carotid IMT in subjects with Type I diabetes [20–22]. We have previously found IMT to be significantly greater in subjects with Type I diabetes with clinical evidence of macrovascular disease than in those without [23]. The fact that the control subjects in the present study were recruited from hospital staff may have introduced some bias, in that health care staff may have healthier lifestyles than the general population. In particular, there were fewer smokers in the control group, although the difference was not statistically significant. This, however, did not confound differences in carotid IMT between the groups; diabetic status remained a significant independent predictor of carotid IMT when age, smoking status and diabetic status were entered into a multiple regression model. Although there were significant associations between carotid IMT and some traditional CHD risk factors, i.e. age and systolic blood pressure, there was no association between carotid IMT and PON1 activity in the present study. Although three studies have demonstrated reduced PON1 activity in subjects who had recently suffered a myocardial infarction [24–26], causality has not been established. It is possible, therefore, that low PON1 activity results from acute myocardial infarction, rather than predisposing a subject to it.

It could be argued that phenyl acetate is not the most appropriate substrate with which to assess PON1 activity, and that a more appropriate method would be to assess a sample’s ability to inhibit LDL oxidation using the *in vitro* model described by Esterbauer et al. [27]. However, decreased PON1 activity measured using phenyl acetate as substrate has been demonstrated following acute myocardial infarction [26]. Phenyl acetate was chosen as the substrate for the measurement of PON1 activity in the present study in order to minimize the risk of genotypic variations at position 192 of the PON1 gene coding region confounding differences in PON1 activity between the groups. However, since the design of the study, another polymorphism of the PON1 coding region, at position 54, as well as three polymorphisms in the PON1 gene promoter region, at positions $-107$, $-824$ and $-907$, have been shown to influence PON1 activity by affecting PON1 mass concentration [28,29]. It is possible, therefore, that genotypic variations between the two groups involving these other sites have had confounding effects on the relative activities of PON1 in the present study.

There were significant differences in HDL composition and particle number between the two groups.
There were greater numbers of the large, more buoyant HDL particles in the subjects with Type I diabetes, accounting for the higher levels of HDL esterified cholesterol and HDL phospholipid. This is consistent with the findings of other studies for subjects with Type I diabetes, which have demonstrated increased HDL cholesterol associated with HDL₃, a less dense HDL subfraction as assessed by sequential ultracentrifugation analysis [30–32]. Furthermore, HDL from Type I diabetic subjects was qualitatively different in the present study, in that there was less triacylglycerol per particle, so that the ratio of esterified cholesterol to triacylglycerol in the core hydrophobic region of the particle was greater. Although other studies have described decreases in the ratio of HDL core esterified cholesterol to triacylglycerol [33,34], the number of subjects in whom this ratio was assessed was small in both of the other studies (eight [33] and six [34] subjects with Type I diabetes). Decreases in lipoprotein triacylglycerol are consistent with increased lipoprotein lipase activity, thought to result from the hyperinsulinaemia associated with the non-physiological subcutaneous systemic delivery of insulin in Type I diabetes [35]. The triacylglycerol content of HDL particles has been shown to determine which of the phospholipase or triacylglycerol lipase activities of hepatic lipase act on them. Triacylglycerol-rich HDL particles are converted by hepatic lipase into smaller particles, which are better substrates for catabolic pathways, whereas phospholipid is removed from the surface of triacylglycerol-poor HDL particles by hepatic lipase without any change in size or density [36]. The reduced triacylglycerol load on HDL from Type I diabetic subjects may therefore make it less likely to undergo catabolism, and could account for the greater numbers of the esterified-cholesterol-laden, large, buoyant HDL particles in the subjects with Type I diabetes. In keeping with this metabolic picture, it has been demonstrated that increased apolipoprotein A1 concentrations in subjects with Type I diabetes are associated with decreases in the fractional catabolic rate of this apolipoprotein [37].

The molar ratio of PON1 activity to apolipoprotein A1 concentration increased progressively in the HDL fractions as particle size decreased and particle density increased. Small HDL particles contain two apolipoprotein A1 molecules, whereas large HDL particles can contain three or four. The molar ratio cannot therefore provide an accurate reflection of the PON1 activity per HDL particle. However, even if a potential halving of the number of apolipoprotein A1 molecules per HDL particle is taken into account when the smaller, denser HDL particles contained in HDL fractions 5–7 are compared with the larger, more buoyant HDL particles contained in HDL fractions 1–3, it can be extrapolated from Figure 5(b) that PON1 activity per HDL particle would still be higher in the smaller, denser HDL particles. It has been demonstrated previously that greater PON1 activity is associated with HDL₃, a more dense HDL subfraction as assessed by sequential ultracentrifugation analysis, than with HDL₂ [38]. However, this finding has not been consistent, and it has also been suggested that PON1 is located in the lower-density region of the HDL spectrum [39]. Prolonged ultracentrifugation can result in qualitative changes in lipoprotein fractions; for example, the loss of apolipoprotein A1 from HDL during prolonged ultracentrifugation is recognized [40,41]. Some studies have found that PON1 dissociates from HDL during prolonged ultracentrifugation, so that PON1 was located in the lipoprotein-free region of the gradient [39]. This effect is unlikely to have contributed significantly to the results of the present study, given the short ultracentrifugation times and the fact that the recovery of PON1 activity in the seven HDL fractions was 87%. For this effect to be significant, it would be necessary for PON1 to dissociate from HDL particles during ultracentrifugation to a greater degree than apolipoprotein A1, and then to appear in more dense HDL fractions post-centrifugation. In other studies, it has proved difficult to separate PON1 from apolipoprotein A1 during ultracentrifugation [10], so that it is unlikely that this effect was contributory in the present study.

The property of the increasing molar ratio of PON1 activity to apolipoprotein A1 concentration as particle size decreases and particle density increases permits speculation as to the HDL compositional determinants of PON1 activity. It has been demonstrated that PON1 associates with HDL by binding surface phospholipids directly [11]. It is possible that the changes in particle size alone contribute to conformational changes in the enzyme that affect its activity. For any spherical particle, as particle size decreases, the proportion of surface constituents to core constituents increases. This is illustrated by the progressive increase in the ratio of the main HDL surface constituent, phospholipid, to the main HDL core constituent, esterified cholesterol, as HDL particle size decreases. It is also possible that the immediate lipid environment influences PON1 activity. For example, the main core lipid, esterified cholesterol, may have an inhibitory effect on the enzyme, so that larger particles, which have greater concentrations of esterified cholesterol relative to phospholipid, to which PON1 binds, have lower PON1 specific activity.

It has been suggested that the ratio of HDL surface phospholipid to unesterified cholesterol affects particle surface fluidity. This may be important in promoting cholesterol efflux from cell membranes, as well as in promoting the activities of lecithin:cholesterol acyltransferase and cholesteryl-ester transfer protein, important steps in reverse cholesterol transport [42–45]. In fact, a decrease in the plasma phospholipid/unesterified cholesterol ratio has been shown to predispose subjects to the development of cardiovascular disease [46]. It is relevant, therefore, that the increase in the ratio of HDL phospho-
lipid to unesterified cholesterol paralleled the progressive increase in the molar ratio of PON1 activity in the fractions. Increased lipoprotein surface fluidity may therefore also increase PON1 specific activity, perhaps by improving access to the substrate. However, since we measured only PON1 activity, rather than activity and mass, it is not possible to differentiate whether changes in PON1 activity reflect changes in specific activity or in PON1 mass. It is therefore also possible that the progressive increase in the molar ratio of PON1 activity to apolipoprotein A1 concentration represents an increase in PON1 mass associated with each HDL particle as particle size decreases and particle density increases. Increased PON1 mass associated with HDL could reflect the increased ratio of surface HDL phospholipid to unesterified cholesterol. Consistent with this is in vitro work involving co-incubation of recombinant HDL particles with a transfected Chinese hamster ovary cell line expressing human PON1. Recombinant HDL particles containing only apolipoprotein A1 and phospholipid acquired more PON1 mass, reflected by increased PON1 concentration and activity, than recombinant HDL particles containing apolipoprotein A1, phospholipid and unesterified cholesterol [47]. In the same study, increased recombinant HDL particle size was associated with more PON1 mass, but only in particles not containing unesterified cholesterol.

In summary, our data argue against an important role for PON1 activity in the predisposition of subjects with Type I diabetes to CHD. There were no correlations between PON1 activity and carotid IMT, the surrogate marker for CHD. Furthermore, there was no difference in PON1 activity between the diabetic and control groups, despite the greater carotid IMT in the subjects with Type I diabetes. We have demonstrated that certain characteristics of the HDL particle are associated with the PON1 activity of that particle. Smaller particle size, and thus a greater ratio of the main surface lipid constituent, phospholipid, to the main core lipid constituent, esterified cholesterol, is associated with higher PON1 activity. An increase in the ratio of HDL phospholipid to HDL unesterified cholesterol is also associated with higher PON1 activity. Despite some significant HDL compositional differences, there were no differences in either the HDL phospholipid/esterified cholesterol ratio or the HDL phospholipid/unesterified cholesterol ratio between the Type I diabetic and control subjects, reflecting the similar PON1 activities in the two groups.

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