

Lipoprotein Subclasses and Particle Sizes and Their Relationship With Coronary Artery Calcification in Men and Women With and Without Type 1 Diabetes

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Type 1 diabetes is associated with increased coronary atherosclerosis, especially in women, even though such patients often have an apparently normal lipid profile. We examined whether lipoprotein particle sizes and subclasses differed between type 1 diabetic subjects ($n = 194$, age 30–55 years) and age- and sex-matched control subjects ($n = 195$). We examined whether any abnormalities were of similar magnitude in men and women. The relationship of particle size to electron beam computer tomography–defined coronary artery calcification, a measure of atherosclerosis, was also examined. Proton nuclear magnetic resonance (NMR) spectroscopy was used to quantify VLDL, LDL, and HDL subclass levels and average particle size on fasting samples. LDL size and subclass were similar in diabetic and nondiabetic men. In contrast, in women diabetes was associated with less large and more small LDL and a reduced LDL size (mean difference 0.2 nm; $P = 0.0009$). This greater effect of diabetes on LDL size in women compared with men was significant ($P = 0.007$). Diabetes was associated with more large and less small HDL and, to a similar degree in both sexes, a higher HDL size (difference of 0.4 nm in men and 0.3 nm in women; both $P < 0.0001$). There were no definitive abnormalities in VLDL size. In nondiabetic subjects, lower average HDL particle size, lower LDL size, and higher VLDL size were significantly associated with coronary calcification ($P = 0.001, 0.02, \text{ and } 0.04$, respectively). Thus the HDL size differences with diabetes would be expected to be antiatherogenic and the LDL size differences proatherogenic. However, there was no clear relationship between particle size and calcification in diabetic subjects. We conclude that in the general population NMR spectroscopy–derived particle size reveals important information about the atherogenicity of lipoprotein pro-

file. Type 1 diabetes is associated with differences in NMR-derived particle size, but their pathogenic significance is unclear. *Diabetes* 51:1949–1956, 2002

Type 1 diabetes is associated with an increased risk of coronary heart disease (CHD). The risk increase is greater in women than men, such that the sex difference in CHD mortality is abolished by diabetes (1,2). This effect is mirrored by a loss of the sex difference in coronary artery calcification (CAC), a measure of atherosclerosis burden, in type 1 diabetic patients (3). Established risk factors do not explain the increased risk of CHD or the loss of the sex difference in CHD in diabetes. Indeed, in the absence of renal failure, and provided glycemic control is reasonable, HDL cholesterol is often higher and triglycerides and LDL cholesterol are often lower in type 1 diabetic than nondiabetic patients (4). An important question is whether, despite apparently normal lipid levels, type 1 diabetic patients may have other abnormalities in the lipoproteins that underlie their increased CHD risk and whether these abnormalities are greater in women than in men. VLDL, LDL, and HDL are each heterogeneous groups comprising particles of varying size. A predominance of smaller LDL particles (termed pattern B) is associated with increased CHD risk (5), and a predominance of smaller HDL particles and large VLDL particles has been associated with increased atherosclerosis at angiography independently of prevailing lipid levels (6). Abnormalities of lipoprotein particle size—e.g., reduced LDL size—have been reported in type 1 diabetic patients with nephropathy (7,8). However, whether such abnormalities are relevant to CHD risk in non-nephropathic patients or to the loss of the sex difference in CHD in diabetic patients is unknown.

Traditional methods for measuring lipoprotein subclasses are labor-intensive, limiting the feasibility of large studies. Proton nuclear magnetic resonance (NMR) spectroscopy provides a reliable and non-labor-intensive method for quantifying the level of lipoprotein subclasses of varying size (9,10). The principle of this method is that lipoproteins of a given diameter emit a characteristic spectral signal, the amplitude of which is proportional to the amount of lipid methyl groups contained in particles of that size range. Using standard assumptions about lipid content of particles of a given size, the levels of VLDL, LDL, and HDL subclasses can be estimated from their

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CAC, coronary artery calcification; CHD, coronary heart disease; CT, computed tomography; CV, coefficient of variation; DCCT, Diabetes Control and Complications Trial; ECG, electrocardiogram; EDIC, Epidemiology of Diabetes Interventions and Complications; HRT, hormone replacement therapy; IDL, intermediate-density lipoprotein; NMR, nuclear magnetic resonance; OR, odds ratio.

NMR signal intensities. We used this method to compare the serum levels of VLDL, LDL, and HDL lipoprotein subclasses in type 1 diabetic patients and a comparison group of nondiabetic men and women aged 30–55 years. Our aims were 1) to compare particle size and lipoprotein subclass distribution in type 1 diabetic patients and control subjects and 2) to examine whether any effect of diabetes on particle size and subclass distribution is greater in women than in men. To assess the clinical significance of any lipoprotein abnormalities, we also examined the relationship of particle size to lipid levels and CAC in these subjects.

RESEARCH DESIGN AND METHODS

Institutional ethics committee approval was obtained, and all subjects gave fully informed written consent. A random sample of type 1 diabetic men and women aged 30–55 years was taken from the diabetes registers of five London hospitals. Type 1 diabetes was defined by age of onset ≤ 25 years and insulin dependence within 1 year of diagnosis. Patients on renal replacement therapy were excluded, but otherwise the sample is representative of diabetic patients of this age. A random sample of the general population, stratified to have a similar age and sex distribution, was drawn from the lists of two London general practices. Of those invited to take part, 53% of diabetic patients (57% of men and 49% of women) and 30% of nondiabetic subjects (31% of men and 30% of women) agreed to take part. These are conservative estimates of response rates, since the London population is highly mobile at this age—many of those written to are likely to have changed address. Subjects were included regardless of any history of heart disease, but only one subject (a diabetic woman) had CHD history (angina). Four nondiabetic subjects with hypertriglyceridemia (>6 mmol/l) were excluded from all analyses. In all, 194 type 1 diabetic patients and 195 nondiabetic men and women were included. **Examination and coronary artery calcification scan.** Respondents completed a standardized questionnaire. Three blood pressure recordings were made after a 5-min rest using an Omron 705c oscillometric device; the mean of the second and third readings was used. Being hypertensive was defined as having a systolic blood pressure ≥ 140 mmHg, a diastolic blood pressure ≥ 90 mmHg, or being on antihypertensive therapy. Obesity was defined as BMI ≥ 30 kg/m². The ratio of waist to hip circumference was also calculated. No retinal examination was carried out, but self-reported history of retinopathy was recorded.

An Ultrafast computed tomography (CT) scanner (C-150XL; Imatron, San Francisco, CA) was used to quantify coronary calcification as previously described (3). Two sets of 20 transverse tomograms of 3-mm thickness were obtained, at 80% of the RR interval, from the lower margin of the bifurcation of the right branch of the pulmonary artery to the apex of the heart, while the subject held his or her breath. A radiologist placed a region of interest around each potentially calcific lesion (peak density >130 Hounsfield units) within the right coronary, circumflex, left anterior descending, and left main coronary arteries. The area and peak density of each lesion was measured. A density score of 1–4 was defined based on the peak density of the lesion, and calcification score was then calculated as the product of the area of the lesion and its density score as described by Agatston et al. (11). To be included in the calcification score, a lesion had to have an area of at least 0.51 mm², i.e., two contiguous pixels, and a peak density of at least 130 Hounsfield units. A total score for each artery and for the entire heart was calculated by summing the lesion scores. The radiation exposure was <1 mSv. All scans were scored by the same radiologist, who was blinded to the sex and diabetes status of the subjects. Based on a small repeatability study ($n = 20$), the within-observer agreement for the presence of any calcification was high ($\kappa = 0.84$).

Laboratory methods. After an overnight fast, blood samples were taken, and total cholesterol, HDL cholesterol, and triglycerides were measured using enzymatic colorimetric methods (12,13). HDL cholesterol was measured directly after stabilization of other lipoproteins (14). LDL cholesterol was calculated using the Friedewald formula. HbA_{1c} was measured using a latex enhanced immunoassay (intra-assay coefficient of variation [CV] 1.7%). Respondents completed two timed overnight urine collections. Women who were menstruating were excluded from urinary albumin analyses (30 nondiabetic women and 24 diabetic women). Urinary albumin was measured using an immunoturbidimetric method (intra-assay CV 2.3%). Microalbuminuria was defined as an albumin excretion rate ≥ 20 μ g/min and <200 μ g/min, and macroalbuminuria was defined as an albumin excretion rate ≥ 200 μ g/min. Apolipoprotein B levels were measured using an immunoturbidimetric method with commercially available kits (Boehringer Mannheim).

Lipoprotein subclass levels were measured on freshly thawed frozen specimens (0.5 ml) using a 400-MHz proton NMR analyzer at LipoMed Inc. (Raleigh, NC). Spectra of each plasma sample were acquired in duplicate at 47°C and the lipid signal envelope at 0.8 ppm deconvoluted to give the amplitudes of the contributing signals from 16 lipoprotein subclasses (10). These subclass signal amplitudes provide a direct measure of the levels of subclass particles present, since the signals emanate in aggregate from the terminal methyl groups of all of the lipids in the particle (cholesterol esters and triglycerides in the particle core, each contributing three methyl groups, and phospholipids and unesterified cholesterol in the surface shell, each contributing two methyl groups). Because lipoproteins are always fully packed with lipid, each particle of a given diameter can be expected to emit a constant size signal, irrespective of lipid compositional differences arising from, for example, variations in the relative amounts of cholesterol ester and triglyceride in the particle core, varying degrees of unsaturation of the lipid fatty acyl chains, or varying phospholipid composition (10). Using empirically measured relations between lipid contents and signal amplitudes of purified VLDL, LDL, and HDL subclass standards isolated from a diverse group of normo- and hyperlipidemic individuals, conversion factors were derived to transform NMR subclass signal amplitudes into subclass particle levels (units of nanomoles per liter for VLDL and LDL subclasses and micromoles per liter for HDL subclasses). The conversion calculations used standard assumptions about the relationship between lipoprotein particle diameter and core volume and the known partial specific volumes of the core lipids (15). We have presented the data in terms of molar levels of lipoprotein particles per liter. For clinical purposes, and in some other publications using the NMR method, using assumptions about the lipid composition of a particle of given size, the particle level data are presented as the amount of lipid carried in particles of that subclass (e.g., cholesterol per deciliter for LDL particles and triglyceride per deciliter for VLDL particles). Because the purpose of this article is partly to shift the focus from the role of lipid disturbances in diabetes to the potential role of disturbances in lipoprotein particle level, size, and subclass distribution, the data are presented here as molar levels of lipoproteins rather than the amount of lipid in the lipoproteins.

Following are the NMR-quantified subclasses and their approximate diameter ranges, plus the mean diameter values used in the particle level calculations: 10 chylomicrons (>200 nm, mean 220 nm): VLDL subclasses—V6 (80–200 nm, mean 140 nm), V5 (60–80 nm, mean 70 nm), V4 (40–60 nm, mean 50 nm), V3 (35–40 nm, mean 38 nm), V2 (31–35 nm, mean 33 nm), and V1 (27–31 nm, mean 29 nm); intermediate-density lipoprotein (IDL) (23–27 nm, mean 25); LDL subclasses—L3 (21.3–23 nm, mean 22 nm), L2 (19.8–21.2 nm, mean 20.5 nm), and L1 (18.3–19.7 nm, mean 19 nm); HDL subclasses—H5 (10–13 nm, mean 11.5 nm), H4 (8.8–10 nm, mean 9.4 nm), H3 (8.2–8.8 nm, mean 8.5 nm), H2 (7.8–8.2 nm, mean 8.0 nm), and H1 (7.3–7.7 nm, mean 7.5 nm). The diameter range estimates were made by calibration to purified subfractions characterized by electron microscopy (VLDL and LDL) or polyacrylamide gradient gel electrophoresis (HDL). IDL and LDL subclass diameters are uniformly ~ 5 nm smaller than those estimated by gradient gel electrophoresis but are consistent with reported electron microscopy (16) and lipid compositional data (17). The HDL subclasses H5, H4, H3, H2, and H1 are similar to the gradient gel electrophoresis subclass designations 2b, 2a, 3a, 3b, and 3c, respectively (18). Also calculated from the NMR data were lipid mass-weighted average particle diameters (in nanometers) of VLDL, LDL, and HDL, obtained by weighting the relative mass percentage of each subclass (as estimated from the amplitude of its methyl NMR signal, rather than particle level) by its diameter. Subjects with predominantly large LDL (so-called pattern A) have average LDL particle sizes of >20.5 nm, and those with mainly small LDL (pattern B) (19) have average LDL sizes of ≤ 20.5 nm. LDL and HDL subclass levels and particle size determined by gradient gel electrophoresis and NMR are highly correlated (10,18), and VLDL subclass data are assessed to be accurate by studies of plasma spiked with varying amounts of VLDL subfractions of defined size obtained by agarose gel chromatography (20). For simplicity of analysis, we combined the various subclasses to define large, small, and intermediate particles (see Table 2). The chylomicron-sized fraction was included with large VLDL because the repeatability for the sum of these subclasses was much higher than for the individual subclasses separately. A repeatability study on blinded duplicate samples showed good repeatability: 0.91 for VLDL particle size, 0.92 for LDL particle size, and 0.96 for HDL particle size. When VLDL particle levels are very few, however, the method has poor analytical accuracy for VLDL subclasses and the VLDL size index can be spuriously high (if a low level of large particles have been detected but no small ones, for example). Thus, differences in VLDL size between those with and without diabetes were also examined when stratified by triglyceride tertile.

Statistical methods. All analyses were carried out using Stata 6 (StataCorp, College Station, TX). The lipoprotein subclass levels and average particle sizes

TABLE 1
Lipid levels and other characteristics in diabetic and nondiabetic men and women

<i>n</i>	Men			Women		
	Nondiabetic 91	Diabetic 101	<i>P</i>	Nondiabetic 104	Diabetic 93	<i>P</i>
Age (years)	37.8 ± 4	38.0 ± 4	0.7	37.9 ± 4	37.5 ± 4	0.5
Triglyceride (mmol/l)	1.32 (1.0–1.7)	1.03 (0.9–1.5)	0.04	0.96 (0.7–1.3)	0.97 (0.7–1.2)	0.9
LDL cholesterol (mmol/l)	3.3 ± 1.1	3.0 ± 0.96	0.03	2.9 ± 0.83	2.8 ± 0.87	0.3
HDL cholesterol (mmol/l)	1.6 ± 0.34	1.7 ± 0.34	0.01	1.8 ± 0.39	2.0 ± 0.49	0.04
ApoB (mg/dl)	99 ± 28	84 ± 24	<0.001	82 ± 20	78 ± 22	0.2
BMI (kg/m ²)	24.9 ± 3.4	25.4 ± 3.2	0.3	25.6 ± 5.6	25.3 ± 3.8	0.6
Waist-to-hip ratio	0.92 ± 0.04	0.91 ± 0.06	0.3	0.81 ± 0.07	0.82 ± 0.07	0.1
HbA _{1c} (%)	5.3 ± 0.4	8.4 ± 1.2	<0.001	5.3 ± 0.4	9.1 ± 1.7	<0.001
Diabetes duration (years)	—	23 ± 7.6	—	—	24 ± 7.4	—
% hypertensive*	19 ± 4	28 ± 5	0.15	6 ± 2	16 ± 4	0.02
% current smoking	27 ± 5	29 ± 5	0.8	24 ± 4	19 ± 4	0.4
% menopausal†	—	—	—	7 ± 2	3 ± 2	0.2
% using hormonal contraception or HRT	—	—	—	23 ± 4	19 ± 4	0.3
% obese	7 ± 3	8 ± 3	0.8	21 ± 4	8 ± 3	0.02
% with albumin excretion rate ≥20 μg/min	3 ± 2	21 ± 4	0.001	3 ± 2	9 ± 3	0.8
% drinking >21 units/week	38 ± 5	25 ± 4	0.04	7 ± 2	11 ± 3	0.3

Data are means ± SD, geometric means (interquartile range), or % (SE). *P* values are for the difference between those with and without diabetes, within each sex, adjusted for age using multiple linear regression. The unadjusted mean values are shown. *Hypertensive is defined as systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg, or on hypertensive therapy. †Menopausal is defined as cessation of menstruation for ≥1 year without other cause.

were compared according to diabetes within each sex using the nonparametric Mann Whitney *U* test, as most of the subclasses and sizes had a skewed distribution. Whether any differences in these lipoprotein measures between diabetic and nondiabetic were independent of other risk factors was further explored using multiple linear regression, to assess the effect of adjusting for other possible explanatory variables. These models included BMI, waist circumference, total units of alcohol consumed per week, pack years of smoking, and systolic blood pressure. An additional set of models also included albumin excretion rate (on those in whom these data were available). For these multivariate analyses, the distribution of subclasses and sizes was normalized using power and log transformations as appropriate. To test whether the difference in lipoproteins between those with and without diabetes differed by sex, we included a diabetes × sex interaction term in these regression models (equivalent to testing whether the sex difference in risk factor distribution differs by diabetes status). Next, Spearman rank correlation coefficients were calculated for the association of subclass levels and particle size with total CAC score. For multivariate analyses, the association of subclass levels and particle sizes with the presence of detectable CAC, defined as a CT Agatston score >0, was examined using logistic regression adjusting for age, sex, and other variables as described below. We chose to dichotomize the data as CAC present/absent and do logistic regression because CAC scores were positively skewed, with a high frequency of zero values, and data transformation would not have normalized this distribution. All subjects were fasting for a mean of 11 h, but those with diabetes had been fasting on average 1 h less. All analyses were repeated adjusting for time elapsed since fasting, and this confirmed the conclusions.

RESULTS

The characteristics of the subjects are shown in Table 1. Compared with nondiabetic men, diabetic men had slightly lower LDL cholesterol and triglyceride and higher HDL cholesterol despite having similar BMI and obesity prevalence. Among women, those with diabetes had a lower prevalence of obesity but there was little difference in triglyceride or LDL cholesterol. HDL cholesterol was slightly higher in diabetic than nondiabetic women, but not after correction for obesity. HbA_{1c} was higher in diabetic women than men (*P* = 0.004). A similar proportion of diabetic (9%) and nondiabetic (12%) women were using hormonal contraception or hormone replacement therapy (HRT), and few were menopausal (defined as cessation of menstruation for at least 1 year without other

cause). Three diabetic subjects and no nondiabetic subjects were on lipid-lowering drugs. Excluding those who were on lipid-lowering drugs, hormonal contraception, or HRT or were menopausal did not alter the conclusions shown below. Of those with diabetes, 16 men and 17 women reported having been told they had retinopathy, of whom 8 and 6, respectively, had had laser therapy. All nondiabetic subjects with albuminuria had microalbuminuria; two diabetic men and two diabetic women had macroalbuminuria. Two nondiabetic men, 3 nondiabetic women, 13 diabetic men, and 13 diabetic women were on blood pressure-lowering drugs.

Lipoproteins by diabetes status and sex. The levels of the lipoprotein subclasses, the total levels of VLDL, LDL, and HDL particles, and the average particle size by diabetes status and sex are shown in Table 2. Because the distribution of many of the subclasses is skewed, the median and 25th and 75th centiles are shown. The *P* values are for the Mann Whitney *U* test of the univariate comparison between diabetic and nondiabetic subjects within each sex.

VLDL. Consistent with their total triglyceride levels, men with diabetes had reduced levels of VLDL particles compared with nondiabetic men. Within each subclass, only the difference in medium VLDL between diabetic and nondiabetic men reached significance. Overall, the relative distribution of these particles across subclasses, as judged by average particle size, was the same for diabetic and nondiabetic men. Women with diabetes had similar levels of VLDL particles as nondiabetic women. Medium VLDL level was significantly lower and the average VLDL size was significantly higher in diabetic than nondiabetic women. The difference in effect of diabetes on VLDL particle size between men and women was significant (*P* = 0.008 for the interaction) and was independent of VLDL particle level (*P* = 0.001 after adjustment). The same data show that there was little sex difference in VLDL particle

TABLE 2
Lipoprotein subclasses and average particle size by diabetes and sex

	Men			Women		
	Nondiabetic	Diabetic	<i>P</i>	Nondiabetic	Diabetic	<i>P</i>
<i>n</i>	91	101		104	93	
Small VLDL (v1,v2) (nmol/l)	47.1 (34–70)	46.3 (29–64)	0.2	35.2 (14–49)	35.3 (14–54)	0.6
Medium VLDL (v3,v4) (nmol/l)	18.1 (8–31)	9.0 (14–23)	0.003	10.3 (5–18)	3.4 (1.5–12)	<0.0001
Large VLDL (v5,v6,chylo) (nmol/l)	1.9 (0.4–5)	1.1 (0.2–3)	0.13	0.8 (0.1–2)	0.6 (0.1–2)	0.8
Total VLDL particles (nmol/l)	74.6 (52–101)	59.7 (42–84)	0.002	51.8 (31–68)	41.8 (18–74)	0.14
VLDL size (nm)	48.2 (45–55)	50.2 (43.5–60.6)	0.4	48.8 (45–53.0)	56.9 (48.4–66.6)	<0.0001
IDL (nmol/l)	0 (0–32)	0 (0–43)	0.4	0 (0–14)	0 (0–158)	0.005
Small LDL (L1) (nmol/l)	583 (217–975)	569 (276–868)	0.4	315 (51–537)	465 (125–743)	0.04
Large LDL (L2,L3) (nmol/l)	951 (743–1,320)	990 (713–1,258)	0.6	1,101 (842–1,296)	928 (647–1,144)	0.002
Total LDL + IDL particles (nmol/l)	1,669 (1,376–2,005)	1,489 (1,270–1,816)	0.05	1,445 (1,225–1,667)	1,446 (1,151–1,706)	0.7
LDL size (nm)	21.0 (20.5–21.3)	21.1 (20.7–21.4)	0.4	21.3 (21–21.6)	21.0 (20.6–21.4)	0.0009
Small HDL (H1,H2) (μmol/l)	18.7 (14–23)	14.8 (11–20)	0.002	12.7 (8–17)	10.7 (7–14)	0.006
Medium HDL (H3) (μmol/l)	4.0 (1.4–9)	3.9 (2–9)	0.84	6.3 (2–10)	5.9 (2–12)	0.88
Large HDL (H4,H5) (μmol/l)	7.8 (6–12)	11.8 (8–15)	<0.0001	12.9 (10–15)	13.8 (11–18)	0.049
Total HDL particles (μmol/l)	33.0 (30–36)	32.3 (30–35)	0.25	31.8 (29–35)	31.8 (28–34)	0.6
HDL Size (nm)	8.8 (8.5–9)	9.2 (8.9–9.5)	<0.0001	9.4 (9–9.7)	9.7 (9.3–10)	0.0001

Data are median (interquartile range). *P* values shown are for the Mann-Whitney *U* test of the comparison between diabetic and nondiabetic subjects within each sex. Chylo, chylomicron.

size in nondiabetic subjects but that VLDL size was greater in diabetic women than men ($P = 0.003$). The difference in particle size between diabetic and nondiabetic women was particularly marked in those in the bottom tertile of triglyceride (15-nm difference in median VLDL size; $P < 0.001$) and was less marked in those in the top two tertiles of triglyceride (5-nm difference in median VLDL size; $P = 0.03$).

IDL. IDL was detectable in 31% of nondiabetic men, 41% of diabetic men ($P = 0.2$ for the diabetic nondiabetic difference), 29% of nondiabetic women, and 45% of diabetic women ($P = 0.02$ for the difference). IDL levels were also significantly higher in diabetic than nondiabetic women but were not different between diabetic and nondiabetic men (Table 2). This greater difference between those with and without diabetes in women compared with men was not significant ($P = 0.5$ for the diabetes \times sex interaction).

LDL. Diabetic men had lower levels of LDL particles than nondiabetic men (borderline statistical significance, $P = 0.05$), but within each of the large and small subclasses, the differences were not significant and average LDL particle size did not differ. The prevalence of pattern B LDL (an average LDL size ≤ 20.5 nm) was 18% in diabetic men and 25% in nondiabetic men ($P = 0.2$). Women with diabetes had similar LDL particle levels to those of nondiabetic women but had significantly more small LDL and less large LDL and a significantly reduced LDL size. Pattern B prevalence was 24% in diabetic women and 7% in nondiabetic women ($P = 0.001$). The difference in the direction of the effect of diabetes on LDL size between men and women was significant ($P = 0.007$ for the diabetes \times sex interaction) and was independent of total LDL particle level and triglyceride level ($P = 0.001$ after adjustment). The same data show that LDL size was significantly greater in nondiabetic women than men ($P < 0.001$), but there was no sex difference in LDL size in the diabetic subjects ($P = 0.7$).

HDL. The total level of HDL particles was not significantly

different in diabetic and nondiabetic subjects in either sex (Table 2). However, both diabetic men and women had a reduced level of small HDL and an increased level of large HDL compared with nondiabetic subjects. Thus HDL particle size was significantly higher in those with diabetes in both sexes, and this difference was independent of HDL particle level and HDL cholesterol ($P = 0.004$ in each sex on adjustment). There was no difference in the effect of diabetes on HDL size in women and men ($P = 0.3$). From the same data, it can be seen that the sex difference in HDL size was of similar magnitude in nondiabetic and diabetic subjects.

Association between lipoproteins and diabetes-specific factors. We examined the relationship of various diabetes-specific factors with subclass levels, total particle levels, and particle sizes in diabetic men and women combined, adjusted for age and sex. Neither diabetes duration nor albuminuria was associated with any of the subclass levels, total particle levels, or particle sizes (all $P > 0.05$). A history of retinopathy was associated with a higher level of small VLDL ($P = 0.02$) and total VLDL particles ($P = 0.008$) and a lower VLDL size ($P = 0.03$). A higher HbA_{1c} was associated with a higher level of small VLDL ($P < 0.0001$), intermediate VLDL ($P < 0.0001$), large VLDL ($P = 0.03$), total VLDL particle level ($P < 0.0001$), small HDL ($P = 0.005$), lower levels of large HDL ($P = 0.004$), and lower HDL size ($P < 0.0001$). The greater HbA_{1c} in diabetic women than men did not explain any of the sex differences in the effect of diabetes on lipoproteins or particle size ($P = 0.006$ and $P = 0.01$ for the diabetes \times sex interaction for VLDL size and LDL size, respectively, on adjustment for HbA_{1c}). A higher insulin dose per unit BMI was associated with a significantly lower LDL size ($P = 0.02$), large HDL level ($P = 0.03$), and HDL size ($P = 0.001$) and with higher levels of intermediate HDL ($P = 0.03$). However, diabetic women had a lower insulin dose per unit BMI than men (1.7 vs. 2.3 units \cdot kg⁻¹ \cdot m⁻²), so insulin dose cannot explain the significantly greater effect

TABLE 3
Spearman correlation coefficient ρ for the association of lipoproteins and particle size with coronary artery calcification score

	Men				Women			
	Nondiabetic (<i>n</i> = 91)		Diabetic (<i>n</i> = 101)		Nondiabetic (<i>n</i> = 104)		Diabetic (<i>n</i> = 93)	
	ρ	<i>P</i>	ρ	<i>P</i>	ρ	<i>P</i>	ρ	<i>P</i>
Small VLDL (v1,v2) (nmol/l)	0.05	0.6	0.1	0.3	-0.01	0.9	0.09	0.4
Medium VLDL (v3,v4) (nmol/l)	0.13	0.2	0.11	0.2	0.18	0.06	-0.02	0.8
Large VLDL (v5,v6,chylo) (nmol/l)	0.27	0.01	0.17	0.1	0.27	0.005	0.04	0.7
Total VLDL particles (nmol/l)	0.13	0.2	0.15	0.1	0.05	0.6	0.11	0.3
VLDL size (nm)	0.11	0.3	0.02	0.8	0.23	0.02	0.06	0.6
IDL (nmol/l)	0.04	0.7	0.11	0.3	0.11	0.3	0.1	0.3
Small LDL (L1) (nmol/l)	0.23	0.03	0.02	0.8	0.19	0.05	0.06	0.6
Large LDL (L2,L3) (nmol/l)	-0.03	0.8	0.08	0.4	0.06	0.6	0.05	0.6
Total LDL + IDL particles (nmol/l)	0.2	0.046	0.13	0.2	0.25	0.009	0.12	0.3
LDL size (nm)	-0.16	0.1	-0.04	0.7	-0.13	0.2	-0.04	0.7
Small HDL (H1,H2) (μ mol/l)	0.1	0.4	0.14	0.2	0.02	0.8	-0.16	0.1
Medium HDL (H3) (μ mol/l)	-0.035	0.7	-0.12	0.2	0.03	0.8	-0.01	0.9
Large HDL (H4,H5) (μ mol/l)	-0.21	0.04	-0.11	0.3	-0.25	0.009	-0.06	0.5
Total HDL particles (μ mol/l)	-0.10	0.3	-0.04	0.7	-0.11	0.2	-0.16	0.1
HDL size (nm)	-0.23	0.02	-0.04	0.7	-0.30	0.002	-0.09	0.4

Chylo, chylomicron.

of diabetes on LDL size in women than men. Women also had a lower dose of insulin per kilogram body weight than men (0.62 vs. 0.73 units/kg).

Role of other risk factors in diabetic differences in lipoproteins. Using multiple regression models, we also examined whether the significant differences in lipoproteins, subclasses, and particle sizes between diabetic and nondiabetic men and women shown in Table 2 were independent of diabetic differences in other risk factors. The models included BMI, waist circumference, systolic blood pressure, alcohol units consumed per week, pack years of smoking, and albumin excretion rate. On adjustment for these factors, the differences between diabetic and nondiabetic men remained. The differences between diabetic and nondiabetic women in small LDL, small HDL, and large HDL were no longer significant. All other differences, including those for LDL and HDL size among women, remained apparent and significant.

Association between lipoproteins and CAC. Table 3 shows the Spearman correlation coefficients for the association of total lipoprotein levels, lipoprotein subclass levels, and average particle size with CAC score. Among diabetic subjects, none of the individual subclass levels or particle sizes were associated with CAC score in either sex. In contrast, among nondiabetic subjects in both sexes, large VLDL, small LDL, and total LDL particle level were positively associated with CAC score, and large HDL and HDL size were inversely associated with CAC score. In nondiabetic women, VLDL size was also positively associated with CAC score. Because the power of these analyses within each of the four groups was limited, we conducted a logistic regression analyses of the relationship of the subclasses and sizes to the presence of any detectable CAC (a score >0), combining men and women but adjusting for sex. As reported previously (3), the prevalence of detectable CAC was similar in diabetic (52%) and nondiabetic (54%; $P = 0.7$) men but was much higher in diabetic (47%) than nondiabetic (21%; $P < 0.001$) women. In the logistic regression analyses, in the nondiabetic group, the

pattern of associations was similar to that for the Spearman correlation coefficients, with detectable CAC being positively associated with large VLDL ($P = 0.005$), small LDL ($P = 0.006$), and total LDL particle level (0.008) and inversely associated with large HDL ($P = 0.02$) and HDL size ($P = 0.001$). In addition, a significant association became apparent between detectable CAC and both VLDL size (a positive association, $P = 0.04$) and LDL size (an inverse association, $P = 0.02$). Among diabetic subjects, using logistic regression to combine men and women and adjust for age, only a higher VLDL particle level was associated with detectable CAC ($P = 0.04$).

As reported previously, BMI was strongly associated with CAC in all four groups (3), so we examined whether the relationships between CAC and lipoprotein subclasses and particle size were independent of BMI. Among nondiabetic subjects, only the association between VLDL size and CAC was independent of BMI ($P = 0.04$ in men and women combined, adjusted for age, sex, and BMI). Among diabetic subjects, on adjusting for BMI, the association between VLDL particle level and CAC was no longer significant ($P = 0.4$), and no other associations were revealed. On adjusting for insulin dose and HbA_{1c} in the diabetic group, the association between VLDL particle level and CAC remained significant ($P = 0.04$), and no other associations with any subclass or particle size were revealed.

Because individual lipoprotein subclass levels are not independent of lipid levels, we examined to what extent associations between subclass and particle size are independent of the relevant lipid levels.

Among nondiabetic subjects, the associations of large VLDL and VLDL size with CAC were not independent of total triglyceride. The associations of small LDL, LDL particle level, and LDL size with CAC were independent of LDL cholesterol ($P = 0.02$, 0.002, and 0.02, respectively, in men and women combined), but only the association of LDL particle level with CAC was also independent of triglyceride ($P = 0.02$). The association between HDL size

and CAC was independent of HDL cholesterol and triglyceride ($P = 0.03$) and also LDL cholesterol ($P = 0.01$). The association between VLDL particle level and CAC in diabetic subjects was not independent of triglyceride. The study was not powered to examine statistical interactions between various risk factors and particle size or subclasses on CAC.

DISCUSSION

As defined by NMR spectroscopy, in men, diabetes was associated with a reduced level of VLDL particles, particularly medium VLDL. There was also a reduced level of small HDL particles and an increased level of large HDL particles, with an unchanged total HDL particle level and an increase in particle size. Diabetes was associated with similar differences in HDL subclass levels and particle sizes in women and men. In women, however, in addition to a reduced level of medium VLDL, there was also an increase in average VLDL particle size with diabetes. Furthermore, in women, diabetes was associated with a higher level of IDL and small LDL and a lower level of large LDL against a background of an unchanged total LDL particle level with a consequent reduction in average LDL particle size. Most of these differences with diabetes were independent of other risk factors. The differences in HDL subclasses and sizes associated with diabetes would be expected to be antiatherogenic. In women, the differences in VLDL size and LDL subclass and size associated with diabetes would be expected to be proatherogenic. Whether this is actually the case is uncertain, however, because we also showed that NMR spectroscopy-defined lipoprotein subclass levels and particle sizes bear a different relationship to coronary artery calcification between diabetic and nondiabetic subjects.

VLDL. Although we found that type 1 diabetic women had an increased average VLDL particle size and that the effect of diabetes on particle size was different in men than women, we consider this to be a spurious finding. The level of large VLDL particles, which was low in all four groups, was actually lowest in type 1 diabetic women. The calculated average VLDL size was higher in diabetic than nondiabetic women simply because their V6 + chylomicron size particle levels, though very low, were slightly higher than those of nondiabetic women (0.17 vs. 0.15 mol/l), and these are weighted very heavily in the calculation of average VLDL size, as the latter is weighted by mass rather than particle level. Thus the inaccuracy of average VLDL size when triglycerides are low might account for the apparently increased VLDL size in diabetic women; consistent with this, the apparent difference in VLDL size was maximal in those in the bottom tertile for triglyceride. Furthermore, VLDL size was not associated with CAC in diabetic patients.

We found that more large VLDL and a higher average VLDL particle size is associated with CAC in nondiabetic subjects, though this effect was not independent of total triglyceride level. In a previous study, NMR spectroscopy-defined large VLDL was independently associated with the extent of atheroma at angiography (6). In contrast, regression of atherosclerosis was more strongly related to reduction in small VLDL particles than that in larger particles in an intervention study (21). It is possible that

circulating large VLDL is directly atherogenic, although its size makes penetration of the vascular wall unlikely. The elevation of triglyceride-rich particles in the postprandial phase is mainly in the large VLDL subclass, and a greater postprandial elevation of chemically measured large VLDL is found in CHD patients than in control subjects (22). Thus an alternative explanation is that in nondiabetic subjects VLDL size might be a sensitive marker of the metabolic consequences of elevated postprandial triglycerides, even when measured in the fasting state. We did not find any relationship between large VLDL or other subclasses or VLDL size and CAC in diabetic patients; indeed, a lower VLDL size was found in patients with retinopathy. Both retinopathy and CAC were associated with higher VLDL particle levels in diabetic patients.

LDL. The difference in the effect of diabetes on LDL subclasses and particle size in men and women was highly significant and was such that the sex difference in LDL size apparent in nondiabetic subjects was absent in diabetic subjects. LDL size was not related to any diabetes-specific factors other than insulin dose per unit BMI. Because insulin dose was higher in men than women, this relation did not explain the loss of the sex difference in LDL size in diabetic subjects. Adjustment for other risk factors did not account for the reduced LDL size in diabetic women, either. Our results contrast with the Diabetes Control and Complications Trial (DCCT)/Epidemiology of Diabetes Interventions and Complications (EDIC) cohort, in which men with diabetes had a lower NMR-defined LDL size than women (23). In type 2 diabetic patients, using other methods for measuring LDL size, diabetic women were found to have a smaller LDL size than diabetic men compared with the general population, and LDL size predicted cardiovascular disease event rates in diabetic women, though not independently of other factors (24,25).

Small LDL have been shown to be more susceptible to oxidation and more atherogenic than larger particles. Smaller LDL or an increase in the proportion of small particles (pattern B) is a component of the so-called atherogenic lipid profile, where it is closely associated with higher triglyceride levels. Consistent with this, in nondiabetic subjects, we confirmed that higher levels of small LDL, lower LDL size, and pattern B LDL were associated with CAC, though not independently of triglyceride level. The association was not independent of BMI either, suggesting that adverse effects on LDL subclasses are part of the pathway by which BMI increases risk of calcification.

At first glance, the increased small LDL and decreased LDL size in diabetic women, with a loss of the sex difference in LDL size, suggests a partial explanation for the observed greater elevation in risk of coronary atherosclerosis in diabetic women than men. However, we found no relationship between small LDL or lower LDL size and CAC among diabetic subjects of either sex. Given the lack of an association between LDL size and CAC in diabetic subjects in our study, we cannot conclude that the alteration of LDL size in diabetic women has a consequence for atherosclerosis risk. In the DCCT/EDIC cohort, lower LDL size was not significantly associated with microvascular complications, although the level of small LDL was higher

in those with retinopathy and nephropathy. We did not find any such relationships.

HDL. In nondiabetic subjects, we found that higher average HDL size was associated with significantly lower CAC; importantly, this effect was independent of HDL cholesterol, triglyceride, and LDL cholesterol. The more large HDL present, the lower the odds of CAC in nondiabetic subjects (odds ratio [OR] for CAC = 0.43 for a 10 $\mu\text{mol/l}$ difference in large HDL; $P = 0.02$). In contrast, small HDL was not associated with CAC (OR for CAC = 0.97 for a 10 $\mu\text{mol/l}$ difference in small HDL; $P = 0.9$). These data add to previous studies suggesting that large HDL particles are more antiatherogenic than smaller HDL particles. Using traditional separation methods for defining HDL subclass or size, the larger (cholesterol rich) HDL_{2b} and HDL_{2a} subclass level was inversely associated with degree of stenosis at angiography, whereas the smaller HDL_{3b} and HDL_{3c} subclasses were positively associated with stenosis (26). Other researchers found less large HDL and more small HDL in CHD cases than control subjects (27). We found that the association between HDL size and CAC was not independent of BMI, consistent with HDL abnormalities being involved in the increased CAC associated with obesity.

Among diabetic patients, higher HbA_{1c} was associated with higher levels of small HDL, lower levels of large HDL, and a reduced HDL size. Despite their glycemia, however, overall diabetic patients had decreased small HDL, increased large HDL, and increased HDL size even beyond that expected from their higher HDL cholesterol levels. The increased HDL size was independent of other risk factors. This finding is in contrast to type 2 diabetes, where HDL cholesterol and size are clearly reduced (28). As with LDL, the origin and composition of these larger HDL particles in type 1 diabetic subjects will determine whether this shift in subclass distribution really is protective against atherosclerosis. We found that there was only weak evidence of any relationship between HDL particle size and CAC in diabetic subjects, and the association was significantly weaker ($P = 0.006$) than in nondiabetic subjects. Thus we cannot state that the increase in large HDL and HDL size in diabetic patients is particularly protective.

Methodological considerations. The NMR spectroscopic analysis method is sensitive only to differing levels of particles of a given size; it is insensitive to chemical compositional differences that may exist in a particular size population of particles in one group of individuals compared with another (10). Although lipoprotein particle size differences are accompanied by compositional differences, primarily in the relative amounts of lipid from the shell and core compartments (29), the reverse is not true. It is therefore possible that there are important compositional differences in diabetes not captured by NMR-defined size that might affect the atherogenicity of a given subclass. This might explain why NMR-defined particle size predicts CAC risk in nondiabetic subjects but not in type 1 diabetes, but this is speculative. Certainly compositional change does occur in diabetes. In other studies, for example in type 1 diabetic patients, the cholesterol content within the small HDL subfraction was increased compared with control subjects (30). In another study, the

free cholesterol:phospholipid ratio within large VLDL was increased in type 1 diabetic patients (31). The free cholesterol:lecithin ratio in HDL was found to be increased in type 1 diabetic women compared with control subjects (31). Some studies have found abnormal lipoprotein composition postprandially (32). Increased glycation and oxidation of lipoprotein components might also result in a different atherogenicity of a given subclass in diabetic patients. However, current theories of the susceptibility of smaller HDL and LDL to oxidation and glycation lead to the expectation that these smaller particles should be more atherogenic in diabetic patients than control subjects. This is not what we found.

An alternative possibility is that CAC may be less strongly associated with CHD in diabetic than nondiabetic patients. We have discussed the validity of CAC as measure of atherosclerosis in diabetes elsewhere (3,33). Briefly, in the only autopsy study of this question, plaques in type 1 diabetic subjects were found to have a similar calcium content for a given amount of plaque as in nondiabetic subjects (34). CAC has been shown to be strongly correlated with clinical CHD in diabetes as defined by history of electrocardiogram (ECG) changes, angina, and infarction (35). The correlation is slightly higher in men than in women, which may partly reflect a different sensitivity of angina and ECG changes for coronary pathology in men than women. It is possible that some of the CAC detected by electron beam CT in diabetic patients is nonatherosclerotic medial calcification, since diabetes is associated with medial calcification in the peripheral vessels. However, the few reports of extensive medial coronary calcification in the literature have been in patients with renal failure, and none of the subjects in this study had renal failure (36). Ultimately, prospective studies are needed to evaluate the predictive value of CAC in diabetes, and these are now underway.

Regarding the potential role of response bias, the response rates differed by diabetes status, but within each stratum, the response rates did not differ by sex. Thus sex differences within each stratum in lipoproteins cannot be explained by response bias, and neither can the different effect of diabetes on lipoproteins in men than women. It is possible that subclass levels may differ between responders and non responders, but there is no reason to suppose that the slope of the relationship between subclasses and CAC would differ with response. Thus response bias is unlikely to explain the lack of a relationship between CAC and lipoprotein subclass or particle size among the diabetic group. Nonetheless it remains possible that stronger or different relationships may have been observed if the response rates had been substantially higher.

In conclusion, these data demonstrate that diabetes affects HDL subclass and increases HDL size independently of lipid levels and that there are sex differences in the effect of diabetes on NMR-defined LDL subclass and particle size. How these diabetes-associated differences in lipoproteins affect atherosclerosis risk in diabetic patients is unclear, as particle size bears little relationship to coronary calcification in diabetic subjects. The data also show that in the general population, NMR spectroscopy-derived particle size reveals important information about the atherogenicity of lipoprotein profile. This is particu-

larly the case for HDL subclasses. NMR spectroscopy methods have important advantages in terms of cost and labor-intensity compared with other methods for examining lipoprotein subclass size, such as gradient gel electrophoresis. However, the data demonstrate that one cannot necessarily assume that the determinants and atherogenicity of lipoproteins of a given size in the general population will apply to diabetic patients. Studies that specifically examine the atherogenic risk conferred by lipoprotein size and composition in diabetic patients are needed. Further investigation is required into the usefulness of NMR spectroscopy-derived lipoprotein particle size information in type 1 diabetic patients.

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