Levels of atherogenic lipoproteins are unexpectedly reduced in interstitial fluid from type 2 diabetes patients[S]

Johanna Apro,[2,*,†,§] Paolo Parini,[**] Anders Broijersén,[1,*] Bo Angelin,[*,†,§] and Mats Rudling[*,†,§]

Metabolism Unit* and KI/AZ Integrated CardioMetabolic Center, Department of Medicine,[†] Karolinska Institutet at Karolinska University Hospital Huddinge, C2-84, S-141 86 Stockholm, Sweden; Molecular Nutrition Unit, Center for Innovative Medicine, Department of Biosciences and Nutrition,[§] Karolinska Institutet at Karolinska University Hospital Huddinge, S-141 86 Stockholm, Sweden; and Division of Clinical Chemistry, Department of Laboratory Medicine,** Karolinska Institutet at Karolinska University Hospital Huddinge, C1-74, S-141 86 Stockholm, Sweden

Abstract **At a given level of serum cholesterol, patients with T2D have an increased risk of developing atherosclerosis compared with nondiabetic subjects. We hypothesized that T2D patients have an increased interstitial fluid (IF)-to-serum gradient ratio for LDL, due to leakage over the vascular wall. Therefore, lipoprotein profiles in serum and IF from 35 T2D patients and 35 healthy controls were assayed using fast performance liquid chromatography. The IF-to-serum gradients for VLDL and LDL cholesterol, as well as for apoB, were clearly reduced in T2D patients compared with healthy controls. No such differences were observed for HDL cholesterol. Contrary to our hypothesis, the atherogenic VLDL and LDL particles were not increased in IF from diabetic patients. Instead, they were relatively sparser than in healthy controls. The most probable explanation to our unexpected finding is that these lipoproteins are more susceptible to retainment in the extravascular space of these patients, reflecting a more active uptake by, or adhesion to, tissue cells, including macrophages in the vascular wall. Further studies are warranted to further characterize the mechanisms underlying these observations, which may be highly relevant for the understanding of why the propensity to develop atherosclerosis is increased in T2D.**—Apro, J., P. Parini, A. Broijersén, B. Angelin, and M. Rudling. **Levels of atherogenic lipoproteins are unexpectedly reduced in interstitial fluid from type 2 diabetes patients.** ***J. Lipid Res.*** **2015.** 56: **1633–1639.**

Supplementary key words apolipoproteins • cholesterol • high density lipoprotein • low density lipoprotein • statins • triglycerides • very low density lipoprotein

T2D is linked to accelerated atherosclerosis and increased risk for premature cardiovascular disease and death. Several mechanisms are likely to contribute to this phenomenon, including the frequent presence of hyperglycemia, dyslipidemia, and inflammation in this condition (1). Although such patients often display elevated serum TGs, reduced HDL, and increased small dense LDL particles, the level of serum LDL cholesterol is generally not markedly elevated. However, LDL cholesterol levels represent a strong predictor of coronary heart disease and stroke in T2D (2–4), and lowering of LDL by pharmacological treatment clearly prevents cardiovascular disease in this condition (5–7). At a given LDL cholesterol level, patients with T2D have an increased risk for atherosclerosis compared with normal (3, 4, 8), a finding that is still poorly understood. Further mechanistic knowledge of this phenomenon should be of great importance for our possibilities to improve prevention of vascular disease, particularly facing the global acceleration of the prevalence of T2D.

Atherosclerosis is thought to be initiated after LDL particles have been translocated from plasma into the subendothelial space of the vascular wall, where they are modified and initiate an inflammatory response (9). In this process, modified LDL particles are engulfed by resident macrophages that when overloaded transform into foam cells. One explanation for why the relative risk of atherosclerosis is increased at a given concentration of LDL in T2D patients compared with nondiabetic subjects may be that there is an increased leakage of lipoproteins over the vascular wall due to endothelial dysfunction in T2D (1). This would enhance the exposure of structures in the subendothelial space, including macrophages, to

This work was supported by the Swedish Research Council, the Stockholm City Council (ALF), the Swedish Heart-Lung Foundation, the NovoNordisk Foundation, the Knut and Alice Wallenberg Foundation, and the Cardiovascular Program at the Karolinska Institute/Stockholm City Council. The authors have nothing to disclose in relation to the content of this study.

Manuscript received 24 February 2015 and in revised form 3 June 2015.

Published, JLR Papers in Press, June 19, 2015
DOI 10.1194/jlr.P058842

Abbreviations: FPLC, fast performance liquid chromatography; HbA1c, glycosylated hemoglobin; hsCRP, high-sensitivity C-reactive protein; IF, interstitial fluid; Lp(a), lipoprotein (a).

[1] Present address of A. Broijersén: Sobi AB, Tomtebodavägen 23A, S-112 76, Stockholm, Sweden.

[2] To whom correspondence should be addressed.
e-mail: Johanna.Apro@ki.se

[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of one table.

atherogenic lipoproteins derived from the circulation. As the permeability of capillaries in skin and other tissues is increased in T2D (10), and because the transfer of macromolecules has been reported to be the same across capillaries and aorta (11), it is reasonable to assume that the permeability of the arteries to lipoprotein particles is likely increased in T2D and that this can be monitored in peripheral interstitial fluid (IF). In accordance with that concept, it has been reported that the initial fractional escape rate of intravenously injected radiolabeled autologous LDL is increased by >30% in patients with T2D compared with healthy controls (12). If this is the case, such patients would be predicted to have a higher IF-to-serum concentration gradient of LDL compared with healthy controls. Due to the technical difficulties to adequately access the IF compartment (13, 14), studies in the metabolism of apoB-containing lipoproteins in IF have been very limited so far, however, and we have not been able to find any published information on the situation in T2D.

In this work, we have tested the hypothesis that the transvascular IF-to-serum gradient of lipoproteins, in particular LDL, is increased in T2D patients compared with healthy controls.

MATERIALS AND METHODS

Pilot study of the effect of statin treatment on lipoprotein IF-to-serum gradient

It was early realized that it would not be practically or ethically feasible to study a statin-free population of T2D patients. Thus, we first performed a pilot study on nondiabetic individuals to explore whether such therapy had any influence on lipoprotein gradient measurements. For this purpose, 19 patients (14 women and 5 men), aged 65 ± 26 years, with peripheral vascular disease and hypercholesterolemia (cholesterol ⩾6.5 mM and/or LDL cholesterol ⩾4.16 mM) were studied. They were treated at the Department of Surgery, Karolinska University Hospital Huddinge, because of intermittent claudication verified by duplex ultrasound or angiography. Patients with angina pectoris, previous acute myocardial infarction, ischemic stroke, or previous coronary artery bypass grafting or percutaneous transluminal coronary angioplasty were excluded. Patients on hypolipidemic and/or aspirin therapies prior to the trial had these withdrawn 4 and 2 weeks before the first treatment period, respectively. They were randomized in a double-blind, crossover fashion to receive either aspirin 320 mg with placebo or aspirin 320 mg with atorvastatin (Lipitor®, Pfizer) 80 mg, once daily. The treatments were given for 4 weeks each, separated by a 4-week washout period when only aspirin were administered. In this pilot study, IF and serum were collected in the fasting state in the same way as in the main diabetes study (see below). The study was approved by the ethics committee at Karolinska Institute, Stockholm, and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained at inclusion.

T2D patients and controls

Altogether, 35 patients with T2D, and 35 age- and gender-matched healthy controls were studied; their clinical characteristics are given in **Table 1**. The study was designed to allow the detection of a 25% increase in IF-to-serum gradient for LDL cholesterol in the patients, with a 95% power at a significance level of 0.05. The patients were recruited from the outpatient clinic of the Department of Endocrinology, Metabolism and Diabetes, Karolinska University Hospital Huddinge, and healthy controls by local advertisements. Laboratory assessment included routine fasting analyses of hemoglobin, glucose, HbA1c, insulin, aspartate aminotransferase, alanine aminotransferase, cystatin C, TGs, and total, HDL, and LDL cholesterol. Blood pressure was measured, and all subjects filled in a form to document their health status and medical history. Control subjects with clinical or laboratory evidence of reduced kidney function, inflammatory disease, vascular disease, thyroid disease, high blood pressure, or treatment for any of those conditions were excluded. For the patients with T2D, exclusion criteria were inflammatory disease and/or oral glucocorticoid treatment. Written informed consent was obtained at inclusion; the study was conducted in accordance with the Declaration of Helsinki and approved by the regional ethics review board in Stockholm.

As expected, diabetic patients had higher body mass index than the controls (Table 1). The average duration of disease was 14 years. The systolic blood pressure was >140 mmHg in 20 of the patients, and cystatin C levels were increased (>1.25 mg/l) in 6 of them. All 35 patients were on long-term antidiabetic treatment (metformin, 23; sulfonylurea, 7; rosiglitazone, 2; pioglitazone, 1; dipeptidyl peptidase inhibitor, 1; glucagon-like peptide 1 (GLP-1) receptor agonists, 3; and/or insulin, 22). Twenty-five of the patients were on lipid lowering therapy (statins, 24; fibrate, 1; ezetimibe, 2).

TABLE 1. Characteristics of T2D patients and controls

	Healthy Controls	T2D Patients	*P*
Males/females (n/n)	27/8	27/8	
Age (years)	61.2 ± 9.8	62.2 ± 10.0	NS
Body mass index (kg/m^2)	25.4 ± 3.1	31.6 ± 5.2	<0.0001
HbA1c (mmol/mol)	37.3 ± 3.5	63.2 ± 18.5	<0.0001
Fasting plasma glucose (mM)	5.2 ± 0.4	8.5 ± 3.1	<0.0001
Blood pressure, systolic (mmHg)	128 ± 9.8	146 ± 14.5	<0.0001
Cystatin C (mg/l)	0.87 ± 0.1	1.04 ± 0.5	NS
hsCRP (mg/l)	1.99 ± 2.92	2.36 ± 2.34	NS
Duration of diabetes (years)	—	13.9 ± 1.4	
Drug treatment against diabetes (n)	—	35	
Lipid-lowering therapy /statin therapy (n/n)	−/−	25/24	
Suffered from one or more myocardial infarctions (n)	—	10	
Pathological cystatin C, >1.25 mg/l (n)	—	6	
Pathological HbA1c, >46 mmol/mol (n)	—	28	

HbA1c, glycosylated hemoglobin; hsCRP, high-sensitivity C-reactive protein. Data are means ± SD. *P* values indicate the significance of difference between the study groups (Student's unpaired *t*-test). One value for HbA1c and one for duration of diabetes are missing from the patients.

Collection of IF and serum

All subjects arrived to the clinic in the morning after overnight fast. Height, weight, and bioimpedance were assessed, and blood pressure was measured in the supine position. Serum hsCRP and cystatin C levels, together with urine protein were determined by routine clinical assays in all subjects. Additional measurements corresponding to the initial screening program were performed in the patients. Suction-blister fluid was generated according to Kiistala (15). Briefly, two plastic chambers (Ventipress Oy, Lappeenranta, Finland) were placed on the skin 5 cm bilaterally to the umbilicus. A mild suction-pressure of 28–32 kPa was generated by a pump (Ventipress Oy) and sustained until blisters were formed (1.5–2 h). Five blisters were formed under each chamber from where IF (250–500 μl) was aspirated with a syringe and stored at −80°C until analysis. Venous blood was collected after 1 h of blister generation, and stored as whole blood, serum, and plasma at −80°C.

Fast performance liquid chromatography analyses

Lipoprotein cholesterol, TGs, and free cholesterol profiles were analyzed by fast performance liquid chromatography (FPLC), as described (16). Briefly, serum (1 μl for cholesterol and free cholesterol, and 2 μl for TGs) and IF (5 μl for cholesterol and free cholesterol, and 20 μl for TGs) lipoproteins were separated on a sepharose column. Reagents (Roche Diagnostics, Mannheim, Germany; and WAKO Diagnostics, Richmond, VA) were continuously added to the eluate online, and absorbance was measured. In the statin study, the volumes added were 2.5 μl serum and 6.7 μl IF. The lipid content in lipoprotein fractions was calculated from the areas under the curves in the elution profiles.

Apolipoprotein and lipoprotein (a) analyses

ELISAs were used to analyze apoAI, apoB (Mabtech, Nacka strand, Sweden), and lipoprotein (a) [Lp(a); Mercodia, Uppsala, Sweden], following manufacturers' instructions. Serum was diluted 1:150,000 (apoAI), 1:5,000 (apoB), and 1:202 [Lp(a)]; and IF 1:30,000 (apoAI), 1:1,000 (apoB), and 1:82 [Lp(a)]. The Lp(a) calibration curve was modified to cover a broader range of concentrations.

Statistical analysis

Values are presented as means ± SD and were log transformed prior to statistical analysis. Statistical analyses were performed with Student's unpaired or paired *t*-test, using GraphPad Prism (GraphPad Software Inc., La Jolla, CA).

RESULTS

In the pilot study, we evaluated the effect of statin treatment on the IF-to-serum gradients for cholesterol and TGs in different lipoprotein particles, using a crossover design. As expected, statin treatment reduced serum levels of VLDL and LDL cholesterol by 60% and 50%, respectively (**Table 2**). In IF, the reductions were 60% and 54%, respectively. Likewise, serum levels of VLDL and LDL TGs were reduced by 45% and 12%, respectively, while their IF levels were reduced by 54% and 28%. Consequently, statin treatment did not change the IF-to-serum gradients for cholesterol or TGs, indicating that the lipoprotein permeability of the vascular wall was unaltered during statin treatment.

On average, serum total cholesterol was 28% lower in diabetic patients, while LDL and HDL cholesterol were 30% and 40% lower, respectively (**Table 3**). A similar pattern, but with more pronounced reductions, was seen in IF, where total and LDL cholesterol was 40% and 44% lower, respectively, in T2D. HDL cholesterol was 36% lower than in healthy controls. Interestingly, and in opposition to the prediction following our original hypothesis, the IF-to-serum gradients for cholesterol were 29% lower for VLDL and 18% lower for LDL in T2D patients compared with controls (**Fig. 1A**). However, the corresponding gradient for HDL did not differ between patients and controls. The diabetic patients on statin treatment had lower serum total and LDL cholesterol levels than those without such treatment, while the groups did not differ in IF-to-serum gradients (supplementary Table 1).

As anticipated, serum total and VLDL TGs were significantly higher (72% and 130%, respectively) in patients than in healthy controls, while total and VLDL TG levels in

TABLE 2. Serum and IF cholesterol and TG and IF-to-serum gradients for cholesterol and TG in placebo- and statin-treated patients with peripheral vascular disease

	Serum (mM)	*P*	IF (mM)	*P*	IF-to-Serum Gradient	*P*
Placebo						
VLDL chol	1.11 ± 0.69		0.12 ± 0.057		0.12 ± 0.066	
LDL chol	4.16 ± 0.61		0.57 ± 0.18		0.14 ± 0.040	
HDL chol	1.42 ± 0.36		0.35 ± 0.12		0.24 ± 0.053	
Total chol	6.69 ± 1.00		1.04 ± 0.26		0.16 ± 0.043	
VLDL TG	1.82 ± 1.32		0.11 ± 0.12		0.060 ± 0.037	
LDL TG	0.49 ± 0.27		0.064 ± 0.031		0.11 ± 0.044	
HDL TG	0.24 ± 0.093		0.043 ± 0.017		0.19 ± 0.071	
Total TG	2.55 ± 1.35		0.22 ± 0.16		0.088 ± 0.036	
Statin						
VLDL chol	0.44 ± 0.35	<0.0001	0.048 ± 0.036	<0.0001	0.15 ± 0.092	NS
LDL chol	2.07 ± 0.42	<0.0001	0.26 ± 0.087	<0.0001	0.13 ± 0.040	NS
HDL chol	1.37 ± 0.38	NS	0.34 ± 0.12	NS	0.25 ± 0.058	NS
Total chol	3.87 ± 0.73	<0.0001	0.66 ± 0.19	<0.0001	0.17 ± 0.045	NS
VLDL TG	1.00 ± 0.77	<0.0001	0.051 ± 0.050	0.0004	0.052 ± 0.024	NS
LDL TG	0.43 ± 0.15	0.0008	0.046 ± 0.014	0.0183	0.11 ± 0.038	NS
HDL TG	0.20 ± 0.076	NS	0.038 ± 0.015	NS	0.19 ± 0.059	NS
Total TG	1.63 ± 0.79	<0.0001	0.14 ± 0.067	0.0011	0.088 ± 0.029	NS

Chol, cholesterol. Data are means ± SD. *P* values indicate the significance of difference from the placebo group (Student's paired *t*-test).

TABLE 3. Serum and IF lipids and apolipoproteins in T2D patients and healthy controls

	Serum	*P*	IF	*P*
Healthy controls				
VLDL chol (mM)	0.52 ± 0.37		0.059 ± 0.042	
LDL chol (mM)	3.43 ± 0.89		0.62 ± 0.24	
HDL chol (mM)	1.50 ± 0.44		0.41 ± 0.14	
Total chol (mM)	5.45 ± 1.04		1.09 ± 0.36	
VLDL TG (mM)	0.77 ± 0.67		0.085 ± 0.075	
LDL TG (mM)	0.44 ± 0.16		0.082 ± 0.032	
HDL TG (mM)	0.18 ± 0.059		0.055 ± 0.019	
Total TG (mM)	1.40 ± 0.72		0.22 ± 0.11	
VLDL free chol (mM)	0.15 ± 0.096		0.030 ± 0.017	
LDL free chol (mM)	1.09 ± 0.24		0.20 ± 0.078	
HDL free chol (mM)	0.41 ± 0.16		0.12 ± 0.050	
Total free chol (mM)	1.65 ± 0.28		0.35 ± 0.12	
ApoAI (g/l)	0.83 ± 0.14		0.19 ± 0.055	
ApoB (g/l)	0.76 ± 0.25		0.28 ± 0.11	
Lp(a) (U/l)	250 ± 340		26 ± 37	
LDL chol/ApoB (mmol/g)	4.65 ± 0.86		2.31 ± 0.53	
HDL chol/ApoAI (mmol/g)	1.79 ± 0.35		2.14 ± 0.30	
T2D patients				
VLDL chol (mM)	0.61 ± 0.40	NS	0.042 ± 0.033	NS
LDL chol (mM)	2.40 ± 0.97	<0.0001	0.35 ± 0.16	<0.0001
HDL chol (mM)	0.90 ± 0.29	<0.0001	0.26 ± 0.11	<0.0001
Total chol (mM)	3.91 ± 1.23	<0.0001	0.65 ± 0.24	<0.0001
VLDL TG (mM)	1.80 ± 1.38	0.0003	0.11 ± 0.098	NS
LDL TG (mM)	0.44 ± 0.15	NS	0.063 ± 0.022	0.0055
HDL TG (mM)	0.17 ± 0.048	NS	0.045 ± 0.016	0.0090
Total TG (mM)	2.41 ± 1.37	0.0002	0.21 ± 0.11	NS
VLDL free chol (mM)	0.17 ± 0.088	NS	0.025 ± 0.018	NS
LDL free chol (mM)	0.78 ± 0.30	<0.0001	0.11 ± 0.045	<0.0001
HDL free chol (mM)	0.23 ± 0.12	<0.0001	0.082 ± 0.036	<0.0001
Total free chol (mM)	1.19 ± 0.36	<0.0001	0.22 ± 0.066	<0.0001
ApoAI (g/l)	0.67 ± 0.14	<0.0001	0.12 ± 0.035	<0.0001
ApoB (g/l)	0.85 ± 0.24	NS	0.12 ± 0.054	<0.0001
Lp(a) (U/l)	360 ± 490	NS	33 ± 40	NS
LDL chol/ApoB (mmol/g)	2.78 ± 0.66	<0.0001	2.88 ± 0.60	0.0001
HDL chol/ApoAI (mmol/g)	1.33 ± 0.29	<0.0001	2.06 ± 0.54	NS

Chol, cholesterol. Data are means ± SD. *P* values indicate the significance of difference from the same fluid in control group (Student's unpaired *t*-test). In serum, values for free cholesterol are missing from three healthy controls, and in IF from one healthy control. One value for serum apoAI in healthy controls is excluded because of being an outlier; consequently, the HDL chol/apoAI ratio is missing. One value each for IF apoB and Lp(a) in healthy controls is missing; consequently, one value for LDLchol/apoB ratio is missing.

IF did not differ between the study groups (Table 3). The level of LDL TGs in IF was 23% lower in T2D patients than in controls, while HDL TGs in IF were 19% lower. As for cholesterol, the IF-to-serum gradients for VLDL and LDL TGs were lower in T2D patients compared with controls, being reduced by 43% and 24%, respectively (Fig. 1B).

Total free cholesterol, and free cholesterol in LDL and HDL were significantly reduced in samples from diabetic patients, in both serum (28, 29, and 44%) and IF (38, 44, and 33%) (Table 3). Furthermore, the IF-to-serum gradients for free cholesterol in VLDL and LDL were reduced by 21% and 20%, respectively, in the patients (Fig. 1C), similar to the IF-to-serum gradients for cholesterol.

The level of apoAI was 19% lower in serum from diabetics compared with controls, and the corresponding level in IF was 34% lower (Table 3). The level of apoB in serum did not differ between patients and controls, while the IF level was 56% lower in the patients. In T2D patients, the IF-to-serum gradient for apoAI was reduced by 19% compared with controls, and the gradient ratio for apoB was reduced by 60%, again indicating a different transcapillary gradient in T2D (Fig. 1D). For Lp(a), no differences were found between the subject groups in either serum or IF levels (Table 3), or in the IF-to-serum gradients (Fig. 1D).

The LDL cholesterol/apoB ratio was calculated as an indicator of LDL particle size. The lower ratio in serum in T2D than in healthy controls indicates that their particles are smaller (Table 3). In contrast, the LDL cholesterol/apoB ratio in IF was larger in the T2D patients than in healthy controls, indicating larger particles in T2D in IF (Table 3). When calculating the HDL cholesterol/apoAI ratio as an indicator of HDL size, the ratio was lower in T2D serum than in healthy controls, indicating smaller HDL in serum in T2D patients (Table 3). There was no difference in HDL cholesterol/apoAI ratio in IF between T2D patients and healthy controls, indicating similar HDL size (Table 3).

DISCUSSION

T2D is a common cause of premature vascular morbidity and death, and there seems to be an inherent increased risk of cardiovascular disease in relation to the presence of established risk factors (3). Thus, although the risk is

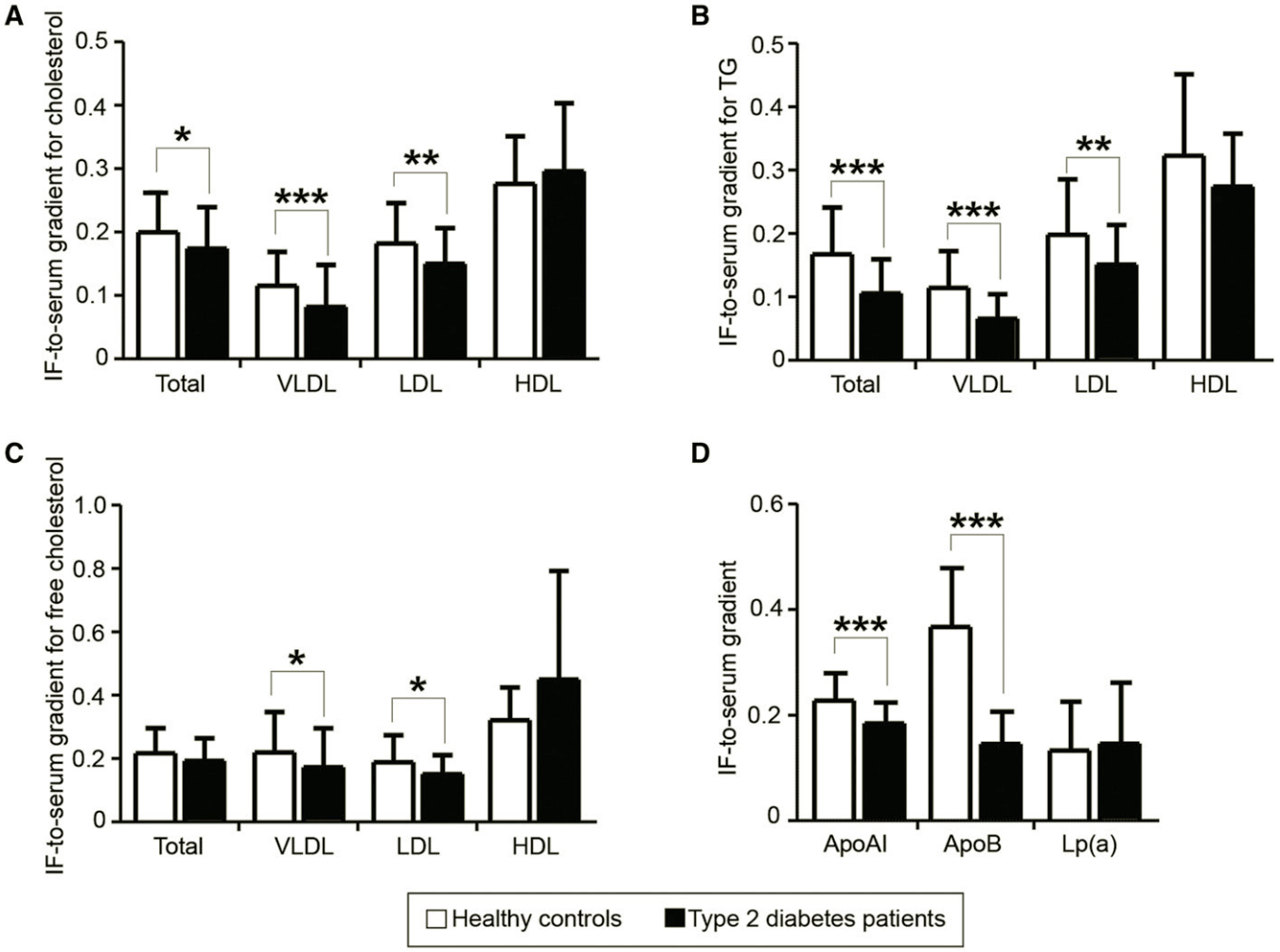

Fig. 1. IF-to-serum gradients for cholesterol, TGs, free cholesterol, and apos in T2D patients and healthy controls. IF-to-serum gradients of lipoprotein cholesterol (A), TGs (B), and free cholesterol (C) in T2D patients and healthy controls analyzed by FPLC. D: IF-to-serum gradients for apoAI, apoB, and Lp(a) in patients and controls. In the diabetes group, the IF-to-serum gradient for VLDL cholesterol and LDL TG are missing for one subject each due to a zero concentration in serum, which makes calculation of the gradient impossible. One value for IF-to-serum gradient for apoAI in healthy controls is excluded because of being an outlier. One value each for IF-to-serum gradient for apoB and Lp(a) in healthy controls is missing. For free cholesterol, the gradients are missing from three subjects. Data are presented as means ± SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Student's unpaired *t*-test).

enhanced with increasing levels of serum LDL cholesterol, the relative risk at a given LDL level is disproportionately elevated in diabetic compared with nondiabetic subjects (3, 4, 8). This is true also when taking the potential influence of other aspects of diabetic dyslipidemia (such as hypertriglyceridemia, low HDL, or the presence of small dense LDL) into account. We here tested the hypothesis that there is an unproportional accumulation of circulating lipoproteins in the subendothelial space in T2D. Such a "leakage" would result in an increased exposure of macrophages in the vascular wall to atherogenic lipoprotein cholesterol, which could explain this apparent anomaly. Unexpectedly, we found that the levels of VLDL and LDL lipoproteins (measured as cholesterol, TG, apoB, or free cholesterol) were instead relatively lower in diabetics than in matched controls.

It is obviously difficult to specifically assess the concentration of lipoproteins in the IF of the vascular wall, which would be the critical location where to fully explore our hypothesis. We used a technique that generates IF in blisters formed by mild suction (15). Such blister fluid has been shown to represent IF (17), but it is clear that our findings have to be taken with caution. There are few reports on lipoprotein levels in IF (13), and the IF-to-serum gradients seen by us are in accordance with those described by others previously (16, 18–20). Our results are also in agreement with the concept that there is a relationship between particle size with IF-to-serum gradient, where the smallest particles display the highest gradient (15, 16, 18, 19). Thus, the relative proportion of the lipoproteins decreases in the order HDL > LDL > VLDL. The apoAI gradient follows that of HDL, and that of apoB essentially that of LDL. The corresponding gradient for Lp(a) has not been described previously, but the finding that its IF-to-serum gradient is considerably lower than for LDL or apoB is reasonable, considering that Lp(a) represents a larger particle than ordinary LDL (21).

Following current treatment recommendations (22), a majority of patients with T2D are on statin therapy. It was therefore essential to first determine if such treatment alters the IF-to-serum gradient of LDL. This was done in a pilot study of hypercholesterolemic patients with claudication, but without symptomatic coronary heart disease. While atorvastatin therapy reduced serum and IF LDL and VLDL cholesterol as well as TGs, there was no change in the concentration gradients of any of the fractions in response to treatment. These data indicate that the exchange between serum and IF is not influenced by variation in the absolute levels of circulating lipoproteins and would also argue against the concept that statins exert some of their beneficial effects on cardiovascular health through changes in this aspect of endothelial function (23, 24).

When comparing the cholesterol levels in T2D patients with those in controls, we found lower levels of total and LDL cholesterol in both serum and IF. These differences are most likely due to concurrent statin treatment, which was present in 24 of the 35 patients. Separate analysis showed that the patients taking statins indeed had lower serum total and LDL cholesterol levels than those who were untreated, but there was no significant difference in IF cholesterol. Of particular importance was the fact that the IF-to-serum gradient did not differ between T2D patients with or without statin treatment (supplementary Table 1), in accordance with the results of our pilot study. Although the fact that a majority of our patients were on lipid-lowering or antidiabetic therapy is a limitation of our study, these findings strongly indicate that our observations are primarily related to the diabetic state itself. The lower serum HDL cholesterol as well as the higher VLDL TGs observed in T2D patients in our study are in agreement with previous observations (25).

The present study demonstrated a clear reduction in the relative concentration of LDL (and VLDL) cholesterol and apoB in IF of T2D patients. In previous studies by Kornerup et al. (12), the early clearance of intravenously injected ^{131}I-labeled LDL was evaluated in T2D, and the disappearance rate of radioactivity was found to be 32% higher compared with controls. This was suggested to be due to an increased transvascular import of LDL particles into the interstitial compartment (12). To explain their findings in relation to our current results, it is plausible to assume that the transfer of LDL into the IF is followed by an enhanced elimination of LDL particles from this compartment in T2D. As the vascular permeability of T2D patients is increased in the whole body, we expect lipoproteins to accumulate in several tissues. The parallel changes seen for VLDL TGs and cholesterol, as well as for apoB, would be compatible with that there is an increased catabolism of apoB-containing lipoproteins within the IF space in T2D. Whether this reflects an enhanced consumption by activated tissue macrophages as part of the atherogenic process, or a stronger retention by the matrix structure within the vascular wall, can only be speculated on at this stage. In this context, it is of interest to note that LDL particles isolated from T2D patients display increased proteoglycan binding (26). Furthermore, it is also shown that subclasses of LDL that display increased proteoglycan binding are also more easily taken up by macrophages (27).

As mentioned previously, T2D patients often display smaller LDL particles. This was confirmed in this study when analyzing LDL cholesterol/apoB ratios, as this ratio in serum in the T2D patients was reduced compared with healthy controls. Interestingly, when analyzing the LDL cholesterol/apoB ratio in IF, the opposite pattern was found (i.e., indications of larger particles in T2D). As smaller particles more efficiently bind proteoglycans (28) one may speculate that small particles in the interstitial compartment in T2D more frequently are bound to proteoglycans and that this could be the reason for why we do not find indications of more smaller particles in T2D than in healthy controls in IF. Moreover, the HDL cholesterol/apoAI ratio in serum was reduced in T2D compared with controls, indicating reduced HDL size in T2D. Because large HDL particles have been shown to reduce the affinity of LDL to proteoglycans (29), the indications of smaller HDL particles in T2D in this material may cause increased proteoglycan binding and contribute to the lower-than-expected IF-to-serum gradient for LDL cholesterol. However, the HDL/apoAI ratio in IF did not differ between T2D patients and healthy controls.

In conclusion, apoB-containing, but not apoAI-containing, lipoprotein particles are sparser in IF relative to their serum levels in patients with T2D. The reduced levels of apoB-containing lipoproteins in IF may mirror an increased uptake of them from IF. The subsequent increased load of cholesterol to peripheral tissues could contribute to the markedly higher propensity to develop atherosclerosis in this disease. Further identification of the mechanisms behind these unexpected findings may lead to important insights that could be relevant in the prevention of accelerated cardiovascular disease in T2D.

The authors thank Catharina Sjöberg, research nurse, for assisting in sample collection; Lilian Larsson, laboratory technician, for assistance with FPLC analyses; and Mabtech AB for providing ELISA assays.

REFERENCES

1. Mazzone, T., A. Chait, and J. Plutzky. 2008. Cardiovascular disease risk in type 2 diabetes mellitus: insights from mechanistic studies. *Lancet.* **371:** 1800–1809.
2. Kothari, V., R. J. Stevens, A. I. Adler, I. M. Stratton, S. E. Manley, H. A. Neil, and R. R. Holman. 2002. UKPDS 60: risk of stroke in type 2 diabetes estimated by the UK Prospective Diabetes Study risk engine. *Stroke.* **33:** 1776–1781.
3. Stamler, J., O. Vaccaro, J. D. Neaton, and D. Wentworth. 1993. Diabetes, other risk factors, and 12-yr cardiovascular mortality for men screened in the Multiple Risk Factor Intervention Trial. *Diabetes Care.* **16:** 434–444.
4. Turner, R. C., H. Millns, H. A. Neil, I. M. Stratton, S. E. Manley, D. R. Matthews, and R. R. Holman. 1998. Risk factors for coronary artery disease in non-insulin dependent diabetes mellitus: United Kingdom Prospective Diabetes Study (UKPDS: 23). *BMJ.* **316:** 823–828.
5. Colhoun, H. M., D. J. Betteridge, P. N. Durrington, G. A. Hitman, H. A. Neil, S. J. Livingstone, M. J. Thomason, M. I. Mackness, V. Charlton-Menys, and J. H. Fuller. 2004. Primary prevention of cardiovascular disease with atorvastatin in type 2 diabetes in the

Collaborative Atorvastatin Diabetes Study (CARDS): multicentre randomised placebo-controlled trial. *Lancet.* **364:** 685–696.
6. Sever, P. S., N. R. Poulter, B. Dahlof, H. Wedel, R. Collins, G. Beevers, M. Caulfield, S. E. Kjeldsen, A. Kristinsson, G. T. McInnes, et al. 2005. Reduction in cardiovascular events with atorvastatin in 2,532 patients with type 2 diabetes: Anglo-Scandinavian Cardiac Outcomes Trial–lipid-lowering arm (ASCOT-LLA). *Diabetes Care.* **28:** 1151–1157.
7. Collins, R., J. Armitage, S. Parish, P. Sleigh, and R. Peto. 2003. MRC/BHF Heart Protection Study of cholesterol-lowering with simvastatin in 5963 people with diabetes: a randomised placebo-controlled trial. *Lancet.* **361:** 2005–2016.
8. Fulcher, J., R. O'Connell, M. Voysey, J. Emberson, L. Blackwell, B. Mihaylova, J. Simes, R. Collins, A. Kirby, H. Colhoun, et al.; Cholesterol Treatment Trialists' (CTT) Collaboration. 2015. Efficacy and safety of LDL-lowering therapy among men and women: meta-analysis of individual data from 174 000 participants in 27 randomised trials. *Lancet.* **385:** 1397–1405.
9. Tabas, I., K. J. Williams, and J. Boren. 2007. Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. *Circulation.* **116:** 1832–1844.
10. Viberti, G. C. 1983. Increased capillary permeability in diabetes mellitus and its relationship to microvascular angiopathy. *Am. J. Med.* **75:** 81–84.
11. Stender, S., and E. Hjelms. 1987. In vivo transfer of cholesterol from plasma into human aortic tissue. *Scand. J. Clin. Lab. Invest. Suppl.* **186:** 21–29.
12. Kornerup, K., B. G. Nordestgaard, B. Feldt-Rasmussen, K. Borch-Johnsen, K. S. Jensen, and J. S. Jensen. 2002. Transvascular low-density lipoprotein transport in patients with diabetes mellitus (type 2): a noninvasive in vivo isotope technique. *Arterioscler. Thromb. Vasc. Biol.* **22:** 1168–1174.
13. Lundberg, J., M. Rudling, and B. Angelin. 2013. Interstitial fluid lipoproteins. *Curr. Opin. Lipidol.* **24:** 327–331.
14. Wiig, H., and M. A. Swartz. 2012. Interstitial fluid and lymph formation and transport: physiological regulation and roles in inflammation and cancer. *Physiol. Rev.* **92:** 1005–1060.
15. Kiistala, U. 1968. Suction blister device for separation of viable epidermis from dermis. *J. Invest. Dermatol.* **50:** 129–137.
16. Parini, P., L. Johansson, A. Broijersen, B. Angelin, and M. Rudling. 2006. Lipoprotein profiles in plasma and interstitial fluid analyzed with an automated gel-filtration system. *Eur. J. Clin. Invest.* **36:** 98–104.
17. Rossing, N., and A. M. Worm. 1981. Interstitial fluid: exchange of macromolecules between plasma and skin interstitium. *Clin. Physiol.* **1:** 275–284.
18. Vermeer, B. J., F. C. Reman, and C. M. van Gent. 1979. The determination of lipids and proteins in suction blister fluid. *J. Invest. Dermatol.* **73:** 303–305.
19. Vessby, B., S. Gustafson, M. J. Chapman, K. Hellsing, and H. Lithell. 1987. Lipoprotein composition of human suction-blister interstitial fluid. *J. Lipid Res.* **28:** 629–641.
20. Tamsma, J. T., F. H. Beverdam, J. A. Leuven, and H. H. Lemkes. 1995. Decreased interstitial apolipoprotein A-I levels in IDDM patients with diabetic nephropathy. *Diabetes.* **44:** 501–505.
21. Fless, G. M., M. E. ZumMallen, and A. M. Scanu. 1986. Physicochemical properties of apolipoprotein(a) and lipoprotein(a-) derived from the dissociation of human plasma lipoprotein (a). *J. Biol. Chem.* **261:** 8712–8718.
22. Stone, N. J., J. G. Robinson, A. H. Lichtenstein, C. N. Bairey Merz, C. B. Blum, R. H. Eckel, A. C. Goldberg, D. Gordon, D. Levy, D. M. Lloyd-Jones, et al. 2014. 2013 ACC/AHA guideline on the treatment of blood cholesterol to reduce atherosclerotic cardiovascular risk in adults: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *Circulation.* **129:** S1–S45.
23. Margaritis, M., K. M. Channon, and C. Antoniades. 2014. Statins as regulators of redox state in the vascular endothelium: beyond lipid lowering. *Antioxid. Redox Signal.* **20:** 1198–1215.
24. Zhang, L., D. Gong, S. Li, and X. Zhou. 2012. Meta-analysis of the effects of statin therapy on endothelial function in patients with diabetes mellitus. *Atherosclerosis.* **223:** 78–85.
25. Mooradian, A. D. 2009. Dyslipidemia in type 2 diabetes mellitus. *Nat. Clin. Pract. Endocrinol. Metab.* **5:** 150–159.
26. Hiukka, A., M. Stahlman, C. Pettersson, M. Levin, M. Adiels, S. Teneberg, E. S. Leinonen, L. M. Hulten, O. Wiklund, M. Oresic, et al. 2009. ApoCIII-enriched LDL in type 2 diabetes displays altered lipid composition, increased susceptibility for sphingomyelinase, and increased binding to biglycan. *Diabetes.* **58:** 2018–2026.
27. Hurt-Camejo, E., G. Camejo, B. Rosengren, F. Lopez, O. Wiklund, and G. Bondjers. 1990. Differential uptake of proteoglycan-selected subfractions of low density lipoprotein by human macrophages. *J. Lipid Res.* **31:** 1387–1398.
28. Anber, V., B. A. Griffin, M. McConnell, C. J. Packard, and J. Shepherd. 1996. Influence of plasma lipid and LDL-subfraction profile on the interaction between low density lipoprotein with human arterial wall proteoglycans. *Atherosclerosis.* **124:** 261–271.
29. Umaerus, M., B. Rosengren, B. Fagerberg, E. Hurt-Camejo, and G. Camejo. 2012. HDL2 interferes with LDL association with arterial proteoglycans: a possible athero-protective effect. *Atherosclerosis.* **225:** 115–120.