Decreased VLDL-Apo B 100 fractional synthesis rate despite hypertriglyceridemia in subjects with type 2 diabetes and nephropathy

Paolo Tessari[1], Edward Kiwanuka[1,2], Rocco Barazzoni[1,3], Gianna M. Toffolo[4], Monica Vettore[1], Irene Cortella[1], Erica Manesso[4], Gloria Pasqualetto[4], Lucia Puricelli[1], Carlo Gabelli[1], Michela Zanetti[1,3]

[1] Dept. of Medicine (DIMED), University of Padova; [2] Diabetic Centre, Treviso; [3] Dept. of Medical, Surgical and Health Sciences, University of Trieste; and [4] Dept. of Information Engineering, University of Padova, Italy.

Context: Subjects with Type 2 Diabetes Mellitus (T2DM) and diabetic nephropathy (DN) often exhibit hypertriglyceridemia. The mechanism(s) of such an increase are poorly known.

Objective: We investigated VLDL-Apo B 100 kinetics in T2DM subjects with and without DN, and in healthy controls.

Design: Stable isotope ^{13}C-leucine infusion, and modelling analysis of tracer-to-tracee ratio dynamics in the protein product pool in the 6–8 hr period following tracer infusion, were employed.

Setting: Male subjects affected by T2DM, either with (n=9) or without (n=5) DN, and healthy male controls (n=6), were studied under spontaneous glycemic levels in the post-absorptive state.

Results: In the T2DM patients with DN, plasma triglyceride (TG) (2.2±0.8 mmol/L, Mean±SD) and VLDL-Apo B 100 (17.4±10.4 mg/dl) concentrations, and VLDL-Apo B 100 pool (0.56±0.29 g), were ~60–80% greater ($p<0.05$ or less) than those of the T2DM subjects without DN (TG: 1.4±0.5 mmol/L; VLDL-Apo B 100: 9.9±2.5 mg/dl; VLDL-Apo B 100 pool: 0.36±0.09 g), and ~80–110% greater ($p<0.04$ or less) than those of nondiabetic controls (TG: 1.2±0.4 mmol/L; VLDL-Apo B 100: 8.2±1.7 mg/dl; VLDL-Apo B 100: 0.32±0.09 g). In sharp contrast however, in the subjects with T2DM and DN, VLDL-Apo B 100 FSR was ≥50% lower (4.8±2.2 pools/day) than that of either the T2DM subjects without DN (9.9±4.3 pools/day, $p<0.025$) or the control subjects (12.5±9.1 pools/day, $p<0.04$).

Conclusions: The hypertriglyceridemia of T2DM patients with DN is not due to hepatic VLDL-Apo B 100 overproduction, which is decreased, but it should be attributed to decreased apolipoprotein removal.

Diabetic nephropathy (DN) in type 2 Diabetes Mellitus (T2DM) is frequently associated with albuminuria, increased concentrations of triglycerides, VLDL-Apo B 100, and fibrinogen, and normal or reduced plasma albumin concentrations (1–3). We have previously shown that in T2DM with DN, both fibrinogen and albumin synthesis are increased (4), the former suggesting a primary defect contributing to the hyperfibrinogenemia (5), likely associated to insulin-resistance (6), the latter unveiling a possible compensatory mechanism for the urinary albumin loss.

The apolipoprotein Apo B 100, fibrinogen and albumin, are three liver-secreted proteins. Whether VLDL-Apo B 100 synthesis is also increased in T2DM with DN, similarly to that of albumin and fibrinogen, thus accounting for the increased circulating concentrations of VLDL-

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA

Received May 4, 2015. Accepted August 13, 2015.

Abbreviations:

Apo B 100 (and triglycerides), has not been established. In previous studies of apolipoprotein kinetics in T2DM, VLDL production was found to be either increased (7) or normal (8). In these studies however, the diabetic subjects were not characterized as regards presence or absence of nephropathy.

The interest into the regulatory mechanisms of these three liver-synthesized proteins arises also from the known association between increased concentrations of triglycerides, VLDL-Apo B 100 and fibrinogen, as well as of urinary albumin excretion, with cardiovascular diseases (9–11).

The apolipoprotein Apo B 100 is a component of both the VLDLs and LDLs. VLDLs predominantly carry circulating triglycerides, whereas LDLs predominantly bind cholesterol and small amounts of triglycerides (12, 13).The study of apolipoprotein kinetics is therefore relevant also with respect to the understading of the mechanisms of the synthesis by the liver of important cardiovascular risk factors.

Therefore, the aim of this study was to measure, in patients affected by T2DM, DN and albuminuria, the rate of VLDL-Apo B 100 synthesis/production, using a leucine-based isotope-dilution method and modeling analysis.

Materials and Methods

Isotope

The L-[1-^{13}C]leucine ([^{13}C]Leu) stable isotope was purchased from Tracer Technologies (Masstrace, Woburn, MA, USA), dissolved in sterile saline under aseptic conditions before use, filtered through a 0.2 μ filter (Millipore, France) and proven to be sterile and pyrogen-free before use.

Patients

Nine male subjects affected by T2DM and DN (here defined as T2DM DN+), five T2DM subjects without DN (defined as T2DM DN-), and six male healthy controls, were recruited. The studies were initiated in 1996 and completed in 2010[1]. The protocol was approved by the Ethical Committee of the Medical Faculty at the University of Padova, Italy, and was performed according to the Helsinki Declaration (1983), as amended in 2008. In the T2DM subjects, DN was diagnosed from an increased albumin excretion rate (AER, >30 mg/d) on the basis of at least two 24-hour determinations, in the absence of other clinical, biochemical and instrumental causes of albuminuria. Three of the T2DM DN+ were microalbuminuric (AER between 30–300 mg/d), while six were macro-albuminuric (AER: >300 mg/d). Six of the T2DM DN+ subjects also had increased plasma creatinine concentrations (>115 μmol/L) (one to 119 μmol/L, four between 135 and 210 μmol/L, the latter to 331 μmol/L).

The clinical and biochemical characteristics of the subjects with T2DM and the controls are reported on Table 1. The T2DM DN+ subjects were modestly older (by 11 and 14 yrs respectively) and heavier (by ~8 and ~13% respectively) than either the T2DM DN- or the non diabetic controls. The overall differences among the three groups were however not significant (p value > 0.07 by ANOVA). All subjects had been adapted to a standard weight-maintaining diet containing ~50% calories as carbohydrates, ~20% proteins and ~30% lipids. Daily protein intake was > 0.8 g/Kg of body weight, unrestricted in all the control and the subjects with T2DM, either without or with microalbuminuria, whereas it was recommended to be at least ≥ 0.6 g/Kg of body weight in the T2DM subjects with macroalbuminuria. The antidiabetic therapy in the T2DM DN+ subjects

Table 1. Clinical and metabolic parameters of the T2DM with albuminuria, and control subjects studied. Means±SD

Parameter	T2DM DN+	T2DM DN-	Controls
Number	9	5	6
Age (years)	56 ± 8	45 ± 10	42 ± 17
BMI (kg/m^2)	31.2 ± 4.5	28.9 ± 3.5	27.7 ± 5.9
Diabetes duration (years)	19 ± 10	14 ± 6	/
Glucose (mmol/liter)	12.3 ± 4.0 [b]	9.5 ± 2.9 [f]	5.1 ± 0.4
Insulin (nmol/liter)	111 ± 24	106 ± 45	82 ± 12
HbA$_1$c (mmol/mmol)	87 ± 16 [b, d]	86 ± 24 [f]	35 ± 3
Total Cholesterol (mmol/liter)	5.36 ± 0.97	4.60 ± 0.76	4.59 ± 0.32
HDL Cholesterol (mmol/liter)	0.86 ± 0.42	1.09 ± 0.26	1.24 ± 0.39
LDL Cholesterol (mmol/liter)	3.68 ± 1.54	3.44 ± 0.96	3.29 ± 0.49
Triglyceride (mmol/liter)	2.19 ± 0.83 [b, c]	1.40 ± 0.51	1.22 ± 0.42
VLDL Apo B (mg/dl)	17.36 ± 10.36 [a, c]	8.44 ± 1.41	8.20 ± 1.70
Albumin (g(L)	37.6 ± 4.9	40.5 ± 2.5	40.7 ± 1.9
Fibrinogen (g/liter)	4.9 ± 1.4 [b, d]	3.1 ± 1.0	2.4 ± 0.4
Creatinine (μmol/liter)	159 ± 76 [b, d]	77 ± 14	77 ± 9

[a]: $P < 0.05$, T2DM DN+ *vs.* Controls; [b]: $P < 0.025$ or less, T2DM DN+ *vs.* Controls;

[c] $P < 0.05$, T2DM DN+ *vs.* T2DM DN-; [d] $P < 0.025$ or less, T2DM DN+ *vs.* T2DM DN-

[e] $P < 0.05$, T2DM DN- *vs.* C; [f] $P < 0.025$ or less, T2DM DN- *vs.* C

consisted, besides diet, of oral hypoglycaemic agents (OHA, n = 2), insulin (n = 3), or combinations of the two (n = 4). Four of the T2DM DN- subjects were treated with OHA, and one with a combination of OHA and insulin. Three T2DM DN+ subjects were also treated with hypolipidemic agents (fibrates). Most of the diabetic patients were taking antihypertensive agents, often in combinations (ACE-inhibitors: n = 7; furosemide: n = 6; hydrochlorotiazide: n = 1; calcium antagonists: n = 9; beta or α-blockers: n = 5; clonidine: n = 1). Five diabetic patients were treated with ticlodipine, one with aspirin. No drug was assumed by any of the control subjects.

Experimental design

On the morning of the study, all subjects were admitted at the Clinical Study Unit of the Metabolism Division of the Padova University Hospital, at 07:00 A.M. A polyethylene catheter was inserted into a dorsal vein of the hand, which was maintained in a plexigas box heated at 55°C, to obtain arterialized-venous blood samples. A second catheter was placed into an antecubital vein of the opposite arm, and used for isotope and dye infusions (see below). Blood samples were taken before the start of the infusions, to determine the baseline natural enrichments of plasma [^{13}C]Leu, [^{13}C]α-ketoisocaproate [^{13}C-KIC], and VLDL-Apo B 100 [^{13}C]Leu.

Thereafter, a primed- (0.6 μmol x kg^{-1}), continuous infusion of [^{13}C]Leu (at the rate of 0.01 μmol x kg^{-1} x min^{-1}) was started, and continued throughout the study using a calibrated pump (Harvard Apparatus, South Natick, MA, USA). Eight ml of whole blood samples were then collected at 30′, 45′, 60′, 90′, 120′, 150′, 180′, 210′ and 240′, and subsequently 60-minute apart between 240′-480′, for the isolation of VLDL-Apo B 100. Additional 5 ml of blood samples were collected *at frequent intervals between 120′ and 480′, for the determination of plasma substrate, isotope and hormone concentrations. In half of the healthy control subjects* the isotope infusion was completed at 360′, since in the first three subjects studied for 480′ the steady state of the VLDL-Apo B 100 [^{13}C]Leu had been attained within the sixth hour.

Plasma volume was calculated from the distribution space of the dye Cardiogreen (14) (Infracyanine, SERB, Paris, France). Briefly, 25 mg of the dye was dissolved in 10 ml of 5% glucose solution and 2 ml of 20% human albumin shortly before use, and rapidly injected within 10 seconds during the first hour of the [^{13}C]Leu infusion, after a brief local reactive hyperemia, obtained by venous stasis, to enhance the systemic delivery of the dye. Plasma samples were taken before and at 1′, 2′, 3′, 4′, 5′, 7′, 10′ and 20′ after the injection. Dye concentration in the infusate as well as in plasma, after proper dilutions, was determined spectrophotometrically at 805 λ. The best fit for a straight line was usually obtained between the 1′ and the 7′-10′ min samples. The intercept of this line in the y axis corresponded to the dye's initial space of distribution (ie, plasma volume).

Analytical methods

Standard biochemical analyses. Plasma glucose, insulin, glycated hemoglobin (HbA_1c), triglycerides, total and HDL cholesterol, creatinine, albumin and fibrinogen concentrations, and daily urinary albumin excretion, were assessed by standard laboratory methods. LDL cholesterol was calculated using the Friedwald's formula.

Isolation of VLDL-Apo B 100, and ^{13}C-Leucine analysis

The lipoproteins were separated from 4 ml plasma by means of standard procedures of preparative sequential ultracentrifugation (15) using a L5–65 ultracentrifuge (Beckman Instruments, Fullerton, CA, USA) and a Ti 50.3 rotor, at densities of 1.006, 1.063 and 1.21 g/mL. The former fraction, containing the VLDLs, was used for further analysis[2.] The protein content of the samples was quantified by the Lowry assay (16). The concentrations of Apo B 100 in the VLDL samples were determined by ELISA (17).

Thereafter, the VLDL fractions were delipidated as described elsewhere (18), then isolated from other proteins (mainly Apo E) using a 3.5%–10% gradient of SDS-PAGE (19), and stained by the Coomassie-Blue. A single band was recovered in correspondence of the theoretical Apo B 100 molecular size, which was further confirmed against commercially-available standard molecular markers. The bands were excised out of the gel, sliced and acid-hydrolyzed for 24 hours. The VLDL-Apo B 100-derived free leucine was then purified using cation exchange chromatography. The leucine and α-ketoisocaproate (KIC) in plasma were isolated as previously described (18), and their [^{13}C]-enrichment, as well as that of [^{13}C]-leucine in the VLDL-Apo B 100 protein-derived hydrolysate, were determined as t-butyldimethylsilyl derivatives by Gas Chromatography-Mass Spectrometry (GCMS) (20), using a Hewlett-Packard GCMS (model 5973), a capillary column and electron impact ionization. Enrichments were expressed as tracer to tracee ratios by using published formulas (21).

Calculations and modeling

The Apo B-100 leucine kinetics in the VLDLs were analyzed using the monoexponential solution of a precursor-product model (22) (see also: Additional data), ie, by assuming that the precursor, that is intracellular [^{13}C]-leucine, rapidly reaches a steady-state condition after the primed continuous infusion.

The tracer incorporation into the product can be described by the following equation:

$$[Eq.1]$$

where: "z(t)" represents the time course of the tracer-to-tracee (TTR) ratio of [^{13}C]-Leu in the VLDL-Apo B-100 protein product pool; "A" is the TTR of the precursor pool, which was that of ^{13}C-Leucine TTR estimated at "plateau" in the VLDL-Apo B 100; "FSR" (min^{-1}) indicates the Fractional Synthesis Rate of VLDL-Apo B 100, which is equal to its fractional turnover since the system was at steady state throughout the experiment; and "τ" (min) is the delay of tracer appearance into the product pool. Notably, the ^{13}C-Leucine TTR estimated at "plateau" in the VLDL-Apo B 100 was very close to that of plasma ^{13}C- α-ketoisocaproate (KIC).

The model parameters A, FSR, and τ, and their precision, were estimated in each individual by applying a weighed least-squares method implemented in MATLAB to the VLDL TTR data. Weights were chosen optimally, ie, equal to the inverse of the measurement errors. They were assumed to be Gaussian, independent, zero-mean and with a constant standard deviation which was estimated a posteriori. (see also: "Additional data").

The absolute secretion rate (ASR, expressed in g x day^{-1}) of

VLDL-Apo B 100 was calculated by multiplying FSR times the plasma (ie, intravascular) protein pool of the protein, on turn calculated from the product of VLDL-Apo B 100 concentrations and the intravascular (ie, plasma) volume, determined from dye bolus-injection data.

Statistical analysis

The data are expressed as Mean±SD. The differences among the groups were analized by the One Way Analysis of Variance (ANOVA) and the Fisher's post hoc test. A p value less than 0.05 was considered as statistically significant.

Power calculation, based on an a priori assumed difference between groups' means of 40%, an estimated SD (the average value of SDs found in previous studies on VLDL-Apo B kinetics, ie, refs #7, #8, and 24#) of 30% of the means, an average number of subjects of ~5 for each group (in our hands they actually were: 5, 6 and 9), and setting alpha at 0.05, yielded a power of 80%, therefore sufficient to detect significant differences among the subjects' groups.

Results

Substrate, hormone and isotope concentrations

The T2DM subjects exhibited greater glucose and HbA_1c levels than the control subjects, whereas plasma insulin concentrations were slightly although not significantly greater than control values (Table 1). Plasma triglyceride, VLDL-Apo B 100, fibrinogen and creatinine concentrations (Table 1) were elevated only in the T2DM DN+ subjects, whereas HDL cholesterol tended to be lower (although insignificantly) in this than in the two other groups. Total and LDL-cholesterol, and albumin concentrations were not different among the groups.

In the T2DM DN+ subjects, the [^{13}C]-Leucine TTR estimated at plateau in the VLDL-Apo B 100 fraction, was ~30% greater (albeit unsignificantly) than that of both the T2DM DN- and the non diabetic subjects (Table 2).

VLDL-Apo B 100 kinetics

The monoexponential model (Eq. 1) resulted to be an adequate description of VLDL-Apo B 100 kinetics, since it was able to fit the TTR data in all our subjects (Figure 1), and its parameters A, FSR, and τ were estimated with acceptable precision (Table 2) (see also: Additional data).

In the T2DM DN+ subjects, the VLDL-Apo B 100 pool size was ~80% greater than that of the two other groups (Figure 2, top). In sharp contrast however, VLDL-Apo B 100 FSR (ie, the number of protein pools turned over in the unit of time), expressed either as min^{-1} (Table 2), or as day^{-1} (Figure 2, middle panel), was ~50%-60% lower than that calculated in both the T2DM DN- and the control subjects. The delay in appearance of VLDL-Apo B 100 into the plasma pool was not different among the groups (Table 2). In the T2DM DN+ subjects, VLDL-Apo B 100 ASR (Figure 2, bottom panel) was ~35% lower than that of the non diabetic controls, whereas in the T2DM DN- subjects it exhibited somewhat intermediate values, without however significant differences among the groups.

Discussion

In this study we show that, in subjects affected by T2DM, diabetic nephropathy and albuminuria, the increased VLDL-Apo B 100 and triglyceride concentrations are not due to an increased VLDL-Apo B 100 fractional synthesis rate, which is rather significantly decreased, with respect to that of both the nondiabetic controls and subjects with T2DM but without nephropathy. Therefore, under the steady-state conditions of this study, the only reasonable explanation for the increased VLDL-Apo B 100 concentration in T2DM subjects with nephropathy is a reduced Apo B 100 removal from plasma.

Our results should be discussed in the context of previous reports investigating Apo B kinetics in T2DM subjects, as well as of the possibile cause(s) for the discrepancies between our and other studies. In the study by Duvillard et al (7), an increased VLDL Apo B 100 production was reported in patients with T2DM. However, there are remarkable differences in subjects' characteristics between that and our study. In the referenced report (7) only six patients with T2DM were studied, they were of both sexes and obese, whereas their non diabetic controls were lean. In the subjects with diabetes, the fasting triglyceride concentrations were markedly increased (by $>$ 5 fold vs. controls), their metabolic control was remarkedly poor (HbA_1c: 9.3%); and the isotopic study

Table 2. Parameters for the calculation of VLDL-Apo B kinetics in the T2DM and control subjects. Values are expressed as Mean±SD Values within brackets indicate the precision of the estimates, as coefficient of variation.

Parameter	T2DM DN+	T2DM DN-	Controls
^{13}C-Leu TTR (estimated at plateau)	0.099 ± 0.027 (31%)	0.077 ± 0.019 (19%)	0.072 ± 0.051 (20%)
VLDL-Apo B FSR (min^{-1})	0.0033 ± 0.0016 [a, b] (51%)	0.0069 ± 0.0030 (39%)	0.0087 ± 0.0063 (42%)
VLDL-Apo B delay (min)	22.3 ± 13.5 (55%)	31.9 ± 16.6 (63%)	24.4 ± 16.1 (52%)

[a]: $P < 0.025$ T2DM DN+ *vs.* Controls; [b]: $P < 0.05$ T2DM DN+ *vs.* T2DM DN-

was performed in the fed state. No data on renal function and albuminuria was reported (7).

In the study by Malmström et al (8), near-normal VLDL Apo B 100 production rates were reported in subjects with T2DM. The subjects with diabetes and the controls there studied were fairly well matched. In the group with diabetes (n = 6), metabolic control was modestly impaired, triglyceride concentrations were increased by only ~30%, and the VLDL-Apo B 100 pool size was normal. That study (8) was performed employing an isotopic method different from ours, ie, using a leucine-tracer bolus injection and a multicompartmental approach for the kinetic analysis. A separate analysis of the VLDL1-and VLDL2 Apo B particles was performed. Furthermore, the study was carried out under both hyperglycemic, hyperinsulinemic-hyperglycemic, and hyperinsulinemic-euglycemic conditions. In the hyperglycemic state (a condition comparable to ours), a normal VLDL1-Apo B, and a decreased VLDL2-Apo B synthesis were reported in the subjects with T2DM, whereas the fractional catabolic rate of these apolipoproteins was slightly decreased in T2DM. No data on the renal status of subjects with diabetes were reported in this study either (8).

In our study, we did not determine the size of the VLDL particles, which are known to be small and dense in patients with T2DM and nephropathy, and associated to hypertriglyceridemia (23). VLDL particles of different size were previously shown to exhibit different kinetic features (8). Whether and to which extent these methodological differences may contribute to the observed discrepancies between previous and the present data, remains to be established.

Interestingly however, in agreement with our findings, also in nondiabetic, nephrotic syndromes (thus exhibiting clinical proteinuria), VLDL-Apo B 100 concentrations were increased whereas their fractional synthesis rates were decreased (24, 25), indirectly suggesting a decreased VLDL-Apo B 100 catabolism as the cause for the hypertriglyceridemia of these subjects. Thus, albuminuria, in either type 2 Diabetes or in non diabetic proteinuric syndromes, seems to carry some specific features associated to a decreased VLDL removal.

As potential explanations of such an hypothesis, several factors should be taken into account. A decreased activity and/or a reduced mass of the lipoprotein lipase (the enzyme removing circulating triglycerides from plasma), had been previously described in diabetic nephropathy (26). This hypothesis was supported also by a greater triglyceride response after an oral fat load in subjects with T2DM nephropathy than in those without nephropathy (26). Ge-

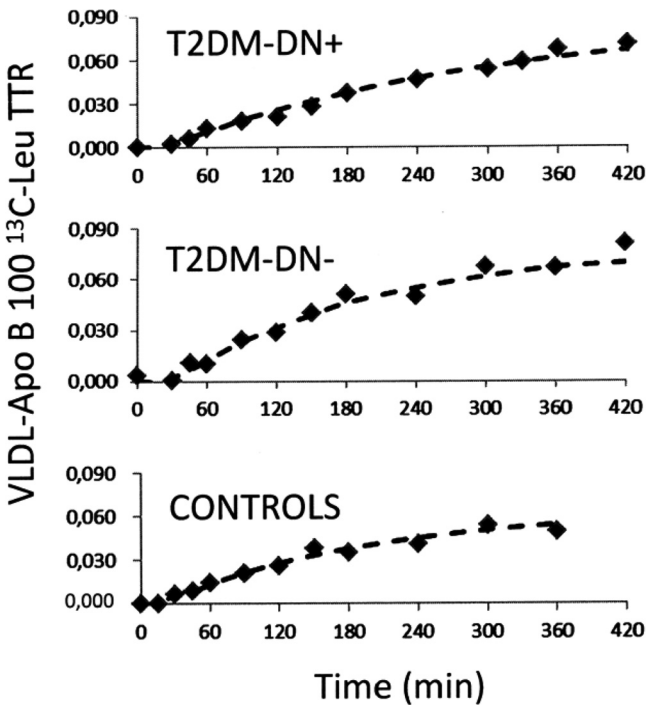

Figure 1. Ability of the monoexponential model (dashed line) to reproduce the VLDL-Apo B 100 tracer-to-tracee data (diamonds) in T2DM-DN+ (upper panel), T2DM-DN+ (middle panel) and Controls (lower panel). Average data vs average model fit are shown.

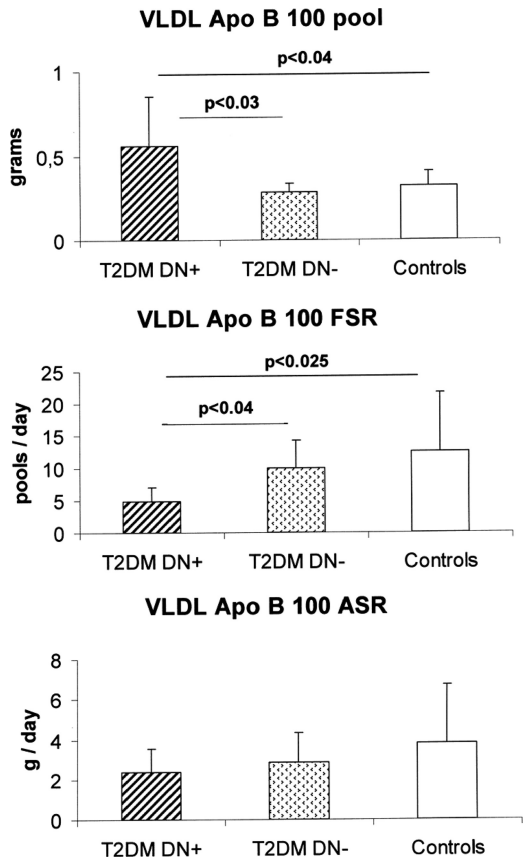

Figure 2. VLDL-Apo B 100 pool (in mg; top panel), fractional synthesis rate (FSR, expressed as day^{-1}; middle panel) and absolute synthesis rate (ASR, in g x day^{-1}; bottom panel) in the type 2 diabetic subjects with diabetic nephropathy (T2DM DN+) (dashed bars), the type 2 diabetic subjects without nephropathy (T2DM DN-) (dotted bars) and in non diabetic control subjects (empty bars).

netic variants of the lipoprotein lipase can be involved too (27). DNA single nucleotide polymorphisms and three common exonic mutations were found to be differentially expressed in T2DM subjects with nephropathy, as compared to normoalbuminuric T2DM subjects (27). Following logistic regression analysis, the P(2)P(2) genotype was independently associated with the presence of microalbuminuria/proteinuria, in addition to systolic blood pressure (BP) and plasma creatinine.

Another possible factors could be represented by endothelial damage, and the associated decreased availability of the lipoprotein lipase (26, 28). Heparin-releasable lipoprotein lipase (LPL) mass was significantly lower in microalbuminuric than in the normoalbuminuric subjects with T2DM, whereas plasma vWF, a marker for endothelial damage, was greater in microalbuminuric than in the normoalbuminuric subjects (28). Furthermore, LPL mass was inversely correlated with plasma vWF level (28).

Finally, proteinuria-associated urinary loss of lipoprotein lipase activators, due to increased glomerular basement membrane permeability, had been suggested as a cause for hyperlipidemia (29).

Taken together, these previously-published, as well as our present data, support the hypothesis of a reduced removal of triglyceride-rich lipoproteins, possibly mediated by a reduction in lipoprotein lipase activity, as the main cause of the hypertriglyceridemia in T2DM subjects with nephropathy. Our kinetic data are consistent with previous work based on measurements of LPL activity, release or circulating mass.

The possible role of insulin resistance should also be discussed. Subjects with type 2 diabetes and nephropathy (ie, with albuminuria) have been reported to be more insulin resistant than subjects without nephropathy (30, 31). Insulin-resistance should lead to an increased hepatic apolipoprotein B-100 production and/or release, due to the loss of the inhibitory effect of insulin on its production (32). In this respect, in T2DM an increased liver production in association with insulin resistance has been reported for fibrinogen, ie, another liver-synthesized proteins (6), but not for albumin (33). In contrast, as regards VLDL-Apo B 100 kinetics, our data are not consistent with a role of insulin resistance on Apo B 100 metabolism, since the VLDL-Apo B 100 fractional production rate in the T2DM subjects with nephropathy was actually decreased, by ~50% with respect to that observed in subjects with T2DM but without nephropathy (Table 2, Figure 2).

HDL cholesterol was decreased in the subjects with T2DM and nephropathy. Although we didn't measured Apo A synthesis, in a previous study Apo A1 fractional catabolism rate was increased in T2DM (34), thus indicating a mechanism for the decreased Apo A1 concentration.

Although the T2DM subjects with DN were 14 and 11 yrs older than either the T2DM DN- or the non diabetic control subjects, respectively, and were also slightly heavier (Table 1), these differences were not statistically significant. Nevertheless, we would exclude any important effect of these variations in the main results of the study. As a matter of fact, we didn't find any correlation between either age or BMI, and VLDL-Apo B 100 FSR ($P > .4$, data not reported). In addition, inclusion of either age or BMI as covariates did not abolish the differences of VLDL-Apo B 100 FSR between the T2DM with DN and the two other groups (data not shown). In any case, visceral obesity, if anything, would rather lead to an increase (not to a decrease) of VLDL Apo B synthesis/secretion, on the basis of observations performed in non diabetic people (35). Age on turn was previously found to be positively correlated with hepatic VLDL Apo B secretion (36–38). As a matter of fact, the greater age and/or BMI values of the T2DM subjects with nephropathy would have rather lead to an underestimation of the differences observed in VLDL Apo B 100 FSR with respect to that of the two other groups.

The results of this study should be discussed also with respect to therapy. Three categories of drugs are currently used to treat hypertriglyceridemia in T2DM: fibrates, statins and omega-3 poly-unsaturated fatty acids (PUFA). Fibrates are peroxisome-proliferator-activated receptor (PPAR)-α agonists, they increase the peripheral clearance of both VLDL- and LDL apo B-100, and reduce VLDL apo B-100 production (39). The same mechanisms are suggested as regards statins, due to their cross-talk with PPARα activators (40), and both inhibition of Apo B 100 production (41) and increase of clearance (42) have been reported with statins. In contrast, omega-3 PUFA affect VLDL metabolism mainly by reducing VLDL triacylglycerol secretion (43). In addition, they may increase both VLDL apoliprotein B degradation (as observed at least in isolated hepatocytes) (44), and (possibly but less certainly) also VLDL clearance, without however affecting the lipoprotein lipase (45). Generally speaking, at least two of the usual lipid-lowering drug cathegories are appropriate to improve the impaired peripheral clearance of VLDL-Apo B containing particles, with the purpose to reduce plasma triglycerides.

In our study, only three of the T2DM patients (one in the normoalbuminuria group, two in the proteinuria group) were treated with a hypolipidemic agent (a fibrate), despite the fact that the triglyceride concentrations were not at target in the latter group as a whole. These agents, as well as other drugs, were withdrawn just the night be-

fore the study. We recognize that this very short wash out period could represent a potential bias. Nevertheless, the individual data of these three subjects fell within 1 SD or less from the corresponding group's mean, and exclusion of these subjects did not change the overall results (data not shown). Furthermore, since fibrates, as stated above, should have increased (ie, not decreased) VLDL-Apo B clearance, their effect should actually have led to an underestimation of the (lowest) values of Apo B clearance in the diabetic group with proteinuria.

As regards the possible role of the hypoglycemic therapy, the T2DM patients with DN were more extensively treated with insulin than those without DN (see: Methods). Since insulin has been reported to decrease Apo B assembly and secretion (46), insulin treatment in the T2DM DN+ subjects might have contributed to suppression of hepatic VLDL-Apo B 100 production. However, the (peripheral) insulin concentrations between the T2DM subjects with and without DN were nearly identical (Table 1), whereas the two groups were clearly different as regards VLDL-Apo B 100 production. In addition, OHA therapy (more extensively used in the T2DM subjects without nephropathy) should stimulate endogenous insulin secretion, thus creating a portal-hepatic vein insulin gradient that, in the presence of comparable peripheral insulin levels, would suggest a greater portal vein insulin concentrations in the OHA vs. the insulin-treated subjects, with a greater effect on the liver. Nevertheless, the T2DM subjects without DN had a greater VLDL-Apo B 100 production than those with DN (Figure 2). Taken together, these observations would exclude a significant effect of the type of hypoglycemic therapy too on the main findings of this study.

Although the severity of nephropathy varied within the T2DM patients with DN, five of them being in the nephrotic range, the creatinine concentrations on average were (modestly) increased in this group, and only in one subjects it was greater than 300 μM. Nevertheless, a possible effect of renal insufficiency itself in the observed findings might strictly not be excluded.

In conclusion, the data of this study indicate a pathophysiological mechanism (ie, a decreased VLDL-Apo B 100 removal) to account for the hypertrygliceridemia of T2DM subjects with diabetic nephropathy, and therefore, they provide a rationale for current treatment protocols aimed at the correction of triglyceridemia in this condition.

Acknowledgments

Address all correspondence and requests for reprints to: Paolo Tessari MD, Dept. of Medicine, Metabolism Division, Via Giustiniani 2, 35 128 Padova, Italy. Phone: +39 049 821/1748/2335., Fax: +39 049 8 754 179. E mail: paolo.tessari@unipd.it.

Disclosure statement: The authors have nothing to disclose.

Authors: P. Tessari: Dept. of Medicine (DIMED), University of Padova, Italy. E mail: paolo.tessari@unipd.it; E. Kiwanuka: Dept. of Medicine (DIMED), University of Padova, Italy. ekiwanuka@ulss.tv.it; R. Barazzoni: Dept. of Medicine (DIMED), University of Padova, and Dept. of Medical, Surgical and Health Sciences, University of Trieste, Italy. e mail: barazzon@units.it; G.M. Toffolo: Dept. Information Engineering, University of Padova, Italy. E mail: toffolo@dei.unipd.it; M. Vettore: Dept. of Medicine (DIMED), University of Padova. E mail: vettore.monica@unipd.it; I. Cortella: Dept. of Medicine (DIMED), University of Padova: E mail irene.cortella@unipd.it.; E. Manesso: Dept. Information Engineering, University of Padova, Italy. E mail: erica.manesso@chem.ethz.ch; L. Puricelli: Dept. of Medicine (DIMED), University of Padova. E mail: lucipuri123@gmail.com; G. Pasqualetto: Dept. Information Engineering, University of Padova, Italy. E mail: glo.pasqualetto@gmail.com; C. Gabelli: Dept. of Medicine (DIMED), University of Padova. E mail: carlo.gabelli@sanita.padova.it; M. Zanetti: Dept. of Medicine (DIMED), University of Padova, and Dept. of Medical, Surgical and Health Sciences, University of Trieste, Italy. E mail: zanetti@units.it

Guarantor: Prof. Paolo Tessari is the guarantor taking responsibility for the contents of the article.

Conflict of interest: None of the authors have any relevant conflict of interest to disclose

[1]The long time span employed for the completion of this study was due to a variety of reasons, such as difficulties in patients' enrolment, the complexity of experiments, measurements and the analyses, and the turnover of the technical personnel trained in lipoprotein separation and isolation, over the years.

[2]The LDL fractions (but not those containing the IDLs) were also processed, and the ^{13}C-leucine enrichment measured by Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS). However, we did not include these data in the MS because of the apparent inadequacy of the experimental design (mostly because of the "short" sampling time span) for the description of LDL kinetics (which is approximately 500-fold slower than that of VLDL Apo B 100).

This work was supported by *Funding/financial support:* This study was supported by Grants of the University of Padova (ex 60%), and of the Italian Minister of University and Research ("PRIN" Grants).

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