ARTÍCULOS DERIVADOS DE ESTA TESIS

- AM Wägner, A Pérez, F Calvo, R Bonet, A Castellví, J Ordóñez. **Apolipoprotein B identifies dyslipidemic phenotypes associated with cardiovascular risk in normocholesterolemic type 2 diabetic patients.** Diabetes Care 1999; 22 (5): 812-17.

- AM Wägner, A Pérez, F Blanco-Vaca, M Rigla, M Cortés, JL Sánchez-Quesada, J Ordóñez-Llanos. **Inaccuracy of calculated LDL cholesterol in type 2 diabetes: consequences for patient risk classification and therapeutic decision**. Clin Chem 2000; 46:1830-32.

- AM Wägner, R Bonet, O Jorba, M Rigla, A Pérez, J Ordóñez-Llanos. **Direct LDL-cholesterol measurement in type 2 diabetic patients.** Enviado a Clin Chem, en segunda revisión.

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- AM Wägner, J Ordóñez-Llanos, R Arcelus, R Bonet, O Jorba, JL Sánchez-Quesada, E Alonso, J Julve, A Pérez. **Postprandial lipidemia in normolipidemic type 2 diabetes: searching for markers.** Enviado. Diabetologia 2001; 44 (Suppl 1): (Abstract) A300.

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Apolipoprotein(B) Identifies Dyslipidemic Phenotypes Associated With Cardiovascular Risk in Normocholesterolemic Type 2 Diabetic Patients

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OBJECTIVE — Apolipoprotein(B) [apo(B)] reflects the total mass of atherogenic particles (VLDL, IDL, and LDL), and its increase is associated with cardiovascular disease independently of LDL cholesterol (LDLc) levels. Apo(B) determination has been recently standardized, but attention to regional reference limits is advisable. Our aim was to analyze the frequency of dyslipidemic phenotypes, including those dependent on increased apo(B) in normocholesterolemic type 2 diabetic patients.

RESEARCH DESIGN AND METHODS — A total of 100 consecutively seen type 2 diabetic patients (63 men, 37 women; aged 59 ± 11 years) were included, after excluding those on lipid-lowering therapy. Apo(B) cutoff (1.1 g/l) was obtained from a group of normolipidemic (47 men, 21 women) control subjects, and LDLc, triglycerides, and HDL cholesterol (HDLc) cutoff points were those from the National Cholesterol Education Program guidelines. LDLc levels were obtained by ultracentrifugation if triglyceride levels were >3.45 mmol/l; otherwise, they were calculated (Friedewald). Apo(B) levels were measured by immunoturbidimetry.

RESULTS — Normocholesterolemia (LDLc <4.13 mmol/l) appeared in 75 of the 100 patients, of whom 55 were normo- and 20 hypertriglyceridemic. Hyperapolipoprotein(B) [hyperapo(B)] was the most frequent lipid disorder, present in 34 (45%) of the normocholesterolemic patients (22 normo- and 12 hypertriglyceridemic). Low HDLc levels were more prevalent (53%) in patients with hyperapo(B) than in the rest (24%).

CONCLUSIONS — Hyperapo(B) was found in almost half of the normocholesterolemic type 2 diabetic patients and was frequently associated with low HDLc levels and hypertriglyceridemia. Thus, given its independent association with cardiovascular disease and that it identifies high-risk phenotypes in normocholesterolemic diabetic patients, apo(B) should be used to evaluate the lipidic pattern of these patients.

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Type 2 diabetes is associated with cardiovascular disease (1,2), which might be due, at least in part, to abnormalities in lipid and lipoprotein metabolism (3,4). Diabetic dyslipidemia comprises multiple lipoprotein disorders. The most typical findings are high triglyceride (Tg) concentrations, low levels of HDL cholesterol (HDLc) and normal or slightly increased LDL cholesterol (LDLc) (5–7). Unlike the near-normalization of lipid levels seen in well-controlled type 1 diabetic patients (8), some degree of lipid alteration persists in type 2 diabetic patients despite improved metabolic control (6,8).

Although LDLc levels are the main therapeutic goal of diabetic and nondiabetic dyslipidemia (9), their concentrations do not stand for the whole mass of lipoproteic particles that also include intermediate-density lipoproteins (IDLs) and VLDLs, whose atherogenicity has been demonstrated (10). Thus, the sole measurement of LDLc levels may underestimate the risk associated with atherogenic lipoproteins. In patients with type 2 diabetes, hypertriglyceridemia reflects an increase in the number and composition abnormalities of VLDL and IDL particles, and it is associated with other atherogenic disorders, such as low HDLc and predominance of small dense LDL particles (3,6,8). Apolipoprotein(B100) [apo(B)] is the principal protein moiety of LDL, IDL, and VLDL, and its concentrations are a good estimate of the total mass of these particles (11). On the other hand, high apo(B) concentration is associated with increased cardiovascular disease, independently of LDLc levels (12–14). Measurement of apoproteins B and AI has gained relevance since an international standard, sponsored by the World Health Organization (WHO) and the International Federation of Clinical Chemistry (IFCC), has become available (15), making transferability of results from different methods theoretically possible. Results from the Framingham Offspring Study have established a cutoff of 1.2 g/l for apo(B) by equiv-

alence to an LDLc level of 160 mg/dl (4.13 mmol/l) (16). However, differences in control populations can exist, and these justify the need to obtain regional population-based reference values for this measure.

Recently, the prospective Quebec Cardiovascular Study showed a higher prevalence of hyperapo(B) with normo- or hypertriglyceridemia in men who developed ischemic heart disease (13,14), and apo(B) levels have shown an association with the number of stenotic coronary vessels in normo- and dyslipidemic women (12). Thus in addition to hypercholesterolemia, low HDLc, and hypertriglyceridemia, hyperapo(B) appears to be a candidate condition to be recognized for the correct evaluation of cardiovascular risk of lipidic origin in patients with type 2 diabetes. Therefore, our aim was to determine the prevalence of dyslipidemic phenotypes, including those depending on apo(B), in a group of normocholesterolemic type 2 diabetic patients, applying the cutoff points recommended by the National Cholesterol Education Program (NCEP) guidelines and those obtained for apo(B) from a normolipidemic control group.

RESEARCH DESIGN AND METHODS

Patient selection
A total of 100 type 2 diabetic patients were consecutively selected from among those seen at the diabetes clinic of our hospital, after discharging those on lipid-lowering drugs or other unrelated drugs or situations known to affect lipoprotein metabolism. Women on hormone replacement therapy were not excluded. The protocol was approved by the ethical committee of our hospital, and patients gave their informed consent.

Diabetes was diagnosed according to the National Diabetes Data Group criteria (17). Smoking was defined as the consumption of one or more cigarettes or other form of tobacco presentation per day; alcohol consumption was defined as the ingestion of one or more alcoholic beverages per week. Patients were considered to suffer from hypertension if they received antihypertensive treatment or their blood pressure was ≥140/90 mmHg in two or more previous visits. Family history of early atherosclerosis was defined as the presence of ischemic heart disease or cerebrovascular events before the age of 55 (men) or 60 (women) years in first-degree relatives.

Table 1—*Clinical and biological features of the diabetic and control subjects involved in the study*

| | | LDLc <4.13 mmol/l | |
	All	Type 2 diabetic patients	Control subjects
n	100	75	68
Age (years)	58.34 ± 11.56	58.7 ± 12.7	56.96 ± 19*
Sex (F/M)	37/63	23/52	21/47
BMI (kg/m²)	28.8 ± 4.7	28.6 ± 4.6	26.58 ± 2.6*
Known diabetes duration (years)	9.85 ± 8.87	9.89 ± 9.3	—
Insulin treatment (%)	55.8	58.6	—
HbA$_{1c}$ (%)	8.1 ± 1.6	8.1 ± 1.7	—
Menopause (%)	84.8	78.2	66.7
Smoking (%)	22.1	24	32.4
Alcohol consumption (%)	16	13.4	—
Known hypertension (%)	53.7	47.1	—
Tc (mmol/l)	5.68 ± 1.02	5.32 ± 0.87	5.11 ± 0.66
Tg (mmol/l)	1.94 ± 1.51	2.0 ± 1.7	1.02 ± 0.4*
HDLc (mmol/l)	1.17 ± 0.34	1.14 ± 0.36	1.35 ± 0.34
LDLc (mmol/l)	3.63 ± 0.82	3.29 ± 0.59	3.29 ± 0.52
VLDLc (mmol/l)	0.78 ± 0.65	0.79 ± 0.73	0.47 ± 0.18*
ApoB (g/l)	1.15 ± 0.21	1.07 ± 0.17	0.95 ± 0.13*

Data are n, means ± SD, or %. *$P < 0.05$ vs. diabetic patients with LDLc < 4.13 mmol/l.

Sudden death by unknown cause was also considered of cardiovascular origin. Peripheric vascular disease was defined by the existence of symptoms consistent with intermittent claudication, absence of distal lower-limb pulses, abnormal Doppler examination, or a history of reconstructive vascular surgery or amputation. Patients who had suffered angina pectoris (retrosternal squeezing or pressure-type discomfort relieved by nitroglycerin and/or accompanied by typical electrocardiographic changes [Minnesota Codes 5-1 or 5-2]) or who had a history of acute myocardial infarction or electrocardiographic signs of necrosis (Minnesota Code 1-1) were considered to suffer from ischemic heart disease. Ischemic cerebrovascular disease was defined by a documented history of stroke. The diagnosis of diabetic retinopathy was established if the assisting physician or ophthalmologist described the presence of microaneurisms, with or without hemorrhages, hard exudates, or new vessels in funduscopic examination with complete pupillary dilatation, or by a history of laser treatment. To assess the presence of diabetic nephropathy, we measured 24-h albumin excretion, after ruling out infection. Microalbuminuria was defined as albumin excretion between 20 and 200 µg/min on two or more occasions, proteinuria or established nephropathy was defined as excretion >200 µg/min, and advanced nephropathy was defined by nephrotic range proteinuria or a creatinine level above reference concentrations (18).

Control subject selection
A group of 68 nondiabetic subjects (47 men, 21 women), defined as normolipidemic according to NCEP guidelines, were selected. They all gave their informed consent, and their clinical and laboratory features are displayed in Table 1.

Methods
Blood samples were obtained after an overnight fast (10–12 h). The blood was left at room temperature for 30 min and the serum was separated by centrifugation (3,000g for 15 min). Total cholesterol (Tc), Tg, HDLc, and apo(B) were immediately analyzed from total serum. For lipoprotein analysis, a preservative solution was added to achieve a final concentration (millimoles per liter) of 1.0 of Na$_2$-EDTA, 0.15 of gentamicine sulfate, 1.2 of chloramphenicol, and 10 of sodium azide (pH 7.2).

Tc and Tg were measured by commercial fully enzymatic methods (Roche Diagnostics, Basel, Switzerland) in the Hitachi 911 analyzer. HDLc was measured by a commercial direct method, without precipitation, using α-cyclodextrin sulphate, MgCl$_2$, and polyethylene-glycol pretreated

cholesterol esterase and oxidase to specifically measure HDLc, even in the presence of the rest of lipoproteins (Roche Diagnostics) (19).

We calculated LDLc by Friedewald's formula (20) when triglyceride did not exceed 3.45 mmol/l (300 mg/dl), and obtained VLDL cholesterol (VLDLc) by dividing total triglyceride (millimoles per liter) by the factor of 2.17 recommended by the NCEP (21). When Tg were ≥ 3.45 mmol/l, we measured LDLc by ultracentrifugation in frozen serum stored at $-80°C$ for no more than 96 h, as is the usual procedure in our lab. In previous work, we have observed that LDLc resulting from Friedewald's equation and ultracentrifugation are interchangeable (in millimoles per liter; LDLc by Friedewald = 0.971 LDL by ultracentrifugation + 0.188; $r = 0.977$) even in populations including 20% of samples with Tg ≥ 3.45 mmol/l (22). Imprecisions of LDLc and VLDLc measures were controlled using pooled sera, whose stability at $-80°C$ we have confirmed for at least 6 months (unpublished data). Between-batch imprecision of 2.9% for LDLc and of 4.3% for VLDLc concentrations was obtained in the range of the samples of the study. Apo(B) was measured by an immunoturbidimetric method (Tina-quant, Roche Diagnostics) using a calibrator whose apo(B) content is standardized against the WHO/IFCC reference standard SP3-07. The imprecision and inaccuracy of the assay were evaluated in 87 replicates during six consecutive months and were found to be 2.3 and 1.6% for concentrations of 0.88 g/l and 2.4 and 3.8% for concentrations of 1.78 g/l, respectively, when assayed by commercial control sera (Precinorm-L and Precipath-L, Roche Diagnostics).

Definition of dyslipidemic phenotypes

Patients with chylomicronemia and suspected dys-β-lipoproteinemia (in millimoles per liter; VLDLc/total Tg ≥ 0.68) were excluded in the first visit. Subjects were considered normolipidemic or dyslipidemic according to the following recommended cutoff points (23): LDLc levels ≥ 4.13 mmol/l (160 mg/dl), Tg ≥ 2.25 mmol/l (200 mg/dl), and HDLc <0.9 mmol/l (35 mg/dl) for men and <1.16 mmol/l (45 mg/dl) for women. We used LDLc and not Tc levels for the definition of hypercholesterolemia to avoid the misclassification as normocholesterolemic of subjects with nor-
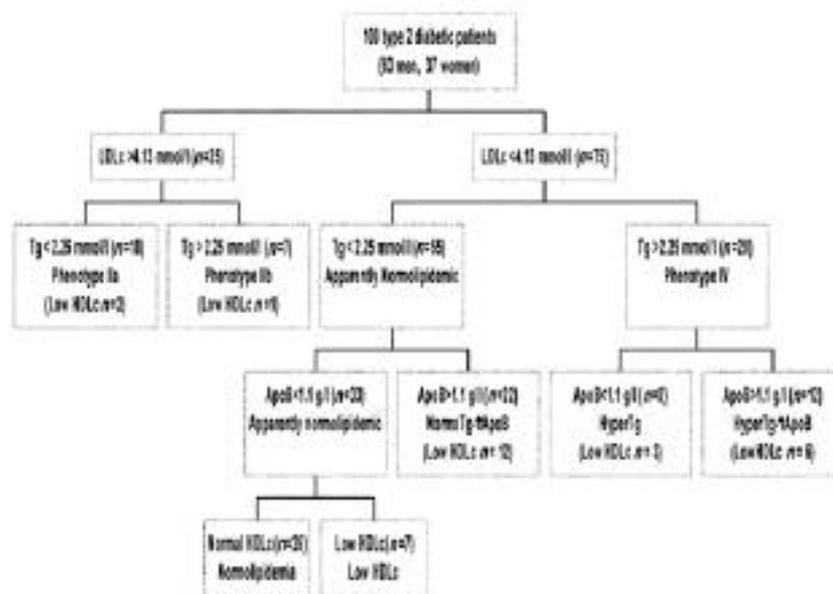

Figure 1—Algorithm used for the classification of dyslipidemic phenotypes among the patients studied and distribution obtained: ↑ApoB, hyperapolipoprotein(B).

mal Tc concentrations as a result of high LDLc and low HDLc levels. The apo(B) cutoff point was calculated according to Contois et al. (16) as the value equivalent to an LDLc value of 4.13 mmol/l in a nondiabetic normolipidemic control group. We obtained the following equation relating apo(B) (grams per liter) and LDLc (millimoles per liter): apo(B) = 0.176 LDLc + 0.377 ($r = 0.712$, $P < 0.001$), and a value of 1.1 g/l resulted for apo(B). These cutoff values were used for sequential classification of the patients into the various dyslipidemic phenotypes, following the algorithm described in Fig. 1. In the first place, patients were classified as normo- or hypercholesterolemic according to their LDLc levels, and then subdivided into phenotypes IIa, IIb, IV, and apparent normolipidemia, depending on their Tg concentrations. Patients showing normal values of LDLc were then subclassified according to apo(B) values. In all the resulting phenotypes, low HDLc was also identified. The remaining subjects were considered to have a normal plasma lipoprotein profile.

Statistical analysis

Analyses were performed using the SPSS 6.0 statistical package for Windows (SPSS, Chicago). Mean ± SD were determined for quantitative data, and frequencies were determined for categorical variables. For continuous variables, and depending on normality distribution, unpaired t or Mann-Whitney U tests were used if comparing two groups, and one-way analysis of variance or Kruskal-Wallis test was used if more than two groups were compared. The χ^2 test and a test for percentage comparison in large samples (24) were used to analyze group differences for categorical variables. The association between continuous variables was tested by linear correlation. All tests were two-tailed, and a P value of ≤ 0.05 was considered significant.

RESULTS — The clinical and biological features of the 63 men and 37 women included in the study are shown in Table 1. The mean of known diabetes duration was 10 years, mean BMI was 29 kg/m², and average glycemic control was poor, despite 55.8% being on insulin treatment. Of the patients, 59% developed microvascular complications (30.4% retinopathy and 44.7% nephropathy with 23% microalbuminuria, 16% proteinuria, and 3% renal failure), and 26% suffered from clinical macrovascular complications (8% cerebrovascular disease, 20% peripheric vascular disease, and 14.7% coronary disease).

Hypercholesterolemia, hypertriglyceridemia, and low HDLc were present in 25, 27 (5% above 3.45 mmol/l), and 31%
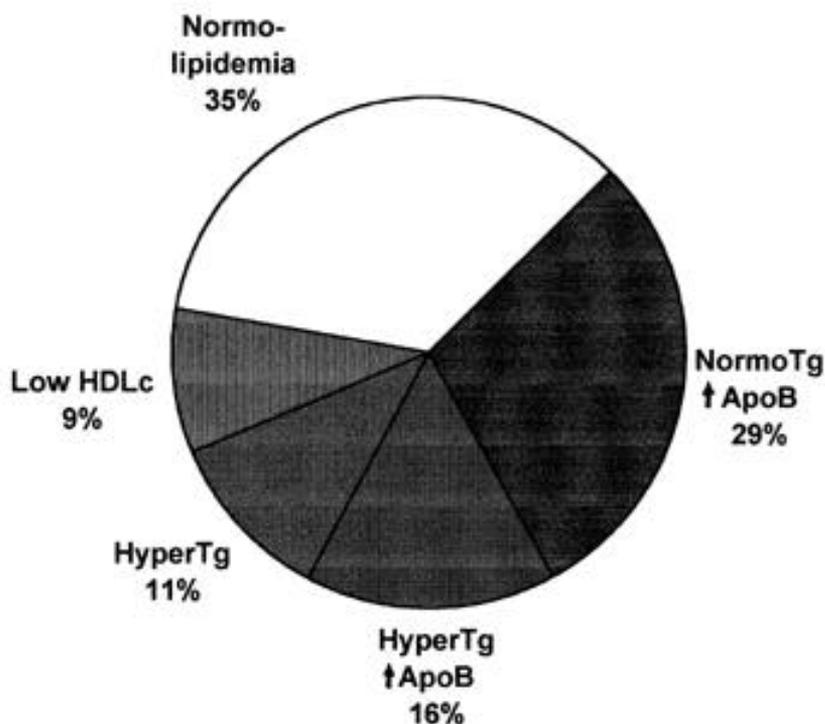
Figure 2—*Distribution of dyslipidemic phenotypes among the normocholesterolemic type 2 diabetic patients included in the study:* ↑*ApoB, hyperapolipoprotein(B).*

of the 100 diabetic patients, respectively. Hyperapo(B) was the most prevalent lipidic abnormality (60% of the whole group), which was present in the 25 patients with hypercholesterolemia, but also in 34 of the 75 normocholesterolemic diabetic patients. Among the control subjects, the frequency of hyperapo(B) was 13%. The mean laboratory results in the diabetic patients and control subjects studied are shown in Table 1. The prevalence of the different dyslipidemic phenotypes, including those dependent on apo(B), in the whole group of diabetic patients, is shown in Fig. 1.

Of the subjects, 18% showed phenotype IIa, and 7% showed phenotype IIb. The distribution of the normocholesterolemic diabetic patients into the different dyslipidemic phenotypes is displayed in Fig. 2. Hyperapo(B) was the most frequent lipid disorder, allowing the identification in apparently normolipidemic ($n = 55$) patients of a subgroup who had hyperapo(B) ($n = 22$) [normotriglyceridemic-hyperapo(B)]. Among the diabetic subjects with phenotype IV ($n = 20$), a subgroup of patients with hyperapo(B) ($n = 12$) [hypertriglyceridemia-hyperapo(B)] was also identified.

Low HDLc levels were more frequently found in hyperapo(B) phenotypes than in the rest (53 vs. 24%, respectively; $P < 0.005$), as shown in Fig. 1.

Finally, no significant differences were found in the clinical features, including insulin treatment, HbA_{1c}, and presence of nephropathy and other long-term diabetic complications, among the patients with the various dyslipidemic phenotypes.

CONCLUSIONS — Increased emphasis is being given to recognizing and treating dyslipidemia in patients with type 2 diabetes and to reduce their high cardiovascular risk (2). The NCEP recommends aggressive treatment with lower goals for serum LDLc in diabetic patients (23). However, the usual lipidic parameters recommended for the evaluation of lipid-related cardiovascular risk (Tg, LDLc, HDLc) do not reflect the total amount of atherogenic particles in these patients. Therefore, additional parameters that do so are needed.

In the present study, we analyzed the impact of the inclusion of apo(B) levels for the classification of type 2 diabetic patients with normal LDLc concentrations into dif-

ferent dyslipidemic phenotypes. The study involved 100 type 2 diabetic patients with a poor average glycemic control. Most of them were referred to the hospital because of difficult clinical management or presence of micro- or macrovascular complications. We excluded subjects treated with lipid-lowering drugs; thus, the prevalence of some of the most easily recognized and frequent dyslipidemic phenotypes (IIa, IIb, IV), as well as patients on secondary prevention caused by macrovascular complications may have been underestimated. However, as already mentioned, our major aim was to analyze normocholesterolemic type 2 diabetic patients.

The distribution of hypercholesterolemia, hypertriglyceridemia, and low HDLc are comparable to those obtained in larger epidemiological studies, despite the different patient selection criteria and cutoff points used (7,25). The cutoff point obtained for apo(B) from our control group, following the procedure used in the Framingham Study, showed a lower value than the latter (1.1 vs. 1.2 g/l, respectively), and proves that regional reference values are still needed, despite international standardization of the measure. According to the cutoff value in our population, hyperapo(B) was the most prevalent (60%) lipidic abnormality. The Framingham Study showed significantly higher levels of apo(B) in diabetic than in nondiabetic women (26). However, to our knowledge, information about the prevalence of hyperapo(B) in type 2 diabetic patients is not available. When we considered the 75 normocholesterolemic diabetic patients, hyperapo(B) remained the most prevalent lipoproteic disorder (45%) and was more frequent than the percentage found in the control group (13%) and in the subjects included in the Quebec Cardiovascular Study, even among those with coronary heart disease. The latter difference might be due to the cutoff point used for the definition of hyperapo(B) (1.35 g/l for the Quebec Study), which was based on a differently standardized apoproteinB immunoassay.

Furthermore, measurement of apo(B) concentrations allowed the identification of a subgroup of apparently normolipidemic and hypertriglyceridemic patients who had hyperapo(B), and thus, increased cardiovascular risk (14). Interestingly, these patients showed a high proportion of low HDLc. Slightly raised Tg, low HDLc, and high apo(B) are metabolically intertwined.

Apo(B) synthesis is required for the hepatic secretion of VLDL, and remains linked to the particle until its clearance from the circulation as IDL or LDL (27). When the catabolism of Tg-rich lipoproteins is impaired, as happens in type 2 diabetes, several lipoproteic disorders associated with hypertriglyceridemia occur, such as increased VLDL remnants, low HDLc, and preponderance of small dense LDL particles (3,8). Hypertriglyceridemia and low HDLc are detected by routine biochemical methods, whereas increased VLDL remnants and the proportion of small dense LDL particles are not. The measurement of plasma apo(B) may be useful to identify these disorders (28). Because there is only one apo(B) molecule per particle (29), measuring plasma apo(B) is roughly equivalent to quantifying the number of apo(B)-containing lipoprotein particles secreted by the liver, mostly LDL particles, that account for ~95% of circulating apo(B) (28). Thus, for a given cholesterol concentration, a high concentration of apo(B) reflects the presence of an elevated number of apo(B)-containing lipoproteic particles (29). In agreement with this statement, we have found that for the same LDLc concentrations (3.29 mmol/l), a 13% higher apo(B) level (1.07 vs. 0.95) is found in normocholesterolemic diabetic patients versus control subjects ($P = 0.045$) (Table 1). The main lipidic difference between these groups is the concentrations of Tg (2.0 vs. 1.0 mmol/l). Thus, total apo(B) concentrations may provide information for a more complete lipidic evaluation than LDLc alone in type 2 diabetic patients.

Finally, the measurement of apo(B) may improve our ability to identify a group of subjects who should be treated, especially those with moderate hypertriglyceridemia, because increased apo(B) levels confer a two to three-fold increase in cardiovascular risk (14). Both HMG-CoA reductase inhibitors (30) and fibrates (31), either alone or combined (32), have been proven to lower apo(B) levels and decrease the number of small dense LDL particles, though only small studies have been performed. Assessment of apo(B) levels might thus also be useful in the evaluation of response to therapy.

In conclusion, measurement of apo(B) concentrations in our study provided information to assess lipidic cardiovascular risk in type 2 normocholesterolemic diabetic patients not obtained from conventional lipoproteic evaluation. Thus although additional prospective studies are necessary to justify its use, bearing in mind its independent association with cardiovascular disease, that it identifies high-risk phenotypes, and that plasma apo(B) measurement has recently been standardized, it seems reasonable to measure apo(B) levels in normocholesterolemic type 2 diabetic patients.

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Inaccuracy of Calculated LDL-Cholesterol in Type 2 Diabetes: Consequences for Patient Risk Classification and Therapeutic Decisions, *Ana Maria Wägner,[1] José Luis Sánchez-Quesada,[2] Antonio Pérez,[1] Mercedes Rigla,[1] Mariano Cortés,[2] Francisco Blanco-Vaca,[2,3] and Jordi Ordóñez-Llanos[2,4*]* (Departments of [1] Endocrinology and Nutrition and [2] Biochemistry, and [3] Research Institute, Hospital de Sant Pau, 08025 Barcelona, Spain; [4] Department of Biochemistry and Molecular Biology, Universitat Autònoma, 08025 Barcelona, Spain; * address correspondence to this author at: Department of Biochemistry, Hospital de Sant Pau, Avgda Sant Antoni Mª Claret 167, 08025 Barcelona, Spain; fax 34-93-2919196, e-mail 2038@hsp.santpau.es)

LDL-cholesterol (LDLc) is the main lipid marker in cardiovascular risk estimation and the principal therapeutic target in both diabetic and nondiabetic subjects *(1, 2)*. The designated comparison method for the determination of LDLc, using ultracentrifugation and precipitation, known as "β-quantification" *(3)*, is cumbersome and time-consuming and requires expensive instrumentation and trained personnel. The Friedewald equation *(4)* {LDLc = total cholesterol − HDLc − [triglycerides (in mmol/L)/2.17 or triglycerides (in mg/dL)/5]}, the most frequently used method for the calculation of LDLc, assumes that VLDL particles maintain a nearly constant cholesterol: triglyceride ratio. However, this assumption is invalid in the presence of chylomicronemia and increased VLDL or intermediate-density lipoprotein particles *(4–7)*.

Because diabetic dyslipidemia includes quantitative and qualitative abnormalities in lipoprotein particles, including VLDL and their remnants *(8–10)*, the use of the Friedewald equation in diabetic patients has been questioned *(11–13)*. HDL-cholesterol (HDLc), often determined after chemical precipitation of apolipoprotein B (apoB)-containing lipoproteins, has technical drawbacks that could interfere with the accuracy of LDLc calculation *(14)*. New homogeneous, direct methods have improved HDLc determination *(15)*. However, the consequences on patient classification and therapy of using direct, more precise methods for HDLc in the estimation of LDLc by the Friedewald equation have, to our knowledge, not been assessed.

We previously proposed an equation that included total triglycerides and cholesterol, and apoB that was more accurate than the Friedewald equation in estimating LDLc *(16)*. Because diabetic dyslipidemia includes hyperapoB *(17)*, an equation that includes apoB in the estimation of LDLc could be of special interest in these patients. Thus, our aims were to ascertain whether a direct HDLc method increases the accuracy of the Friedewald formula, to evaluate an equation that includes apoB in the estimation of LDLc, and to assess the proportion of patients misclassified by the different equations and the therapeutic consequences of that misclassification in type 2 diabetic patients. Comparisons were made against β-quantification.

Ninety-five consecutive nonchylomicronemic type 2 diabetic patients (61% male; age, 57.7 ± 10.7 years,

mean ± SD), with a mean diabetes duration of 10 years (range, 0–33 years) and mean glycohemoglobin of 7.9% (5.7–14%) were studied; 58% received insulin therapy, 54% had microangiopathy, and 31% had macroangiopathy. Dysbetalipoproteinemia was ruled out by a VLDL-cholesterol/total triglyceride ratio >0.65. Dyslipidemia was defined using the following cutoff points: 2.25 mmol/L for total triglycerides and 4.13 mmol/L for LDLc *(2)*. HyperapoB was defined according to a previously obtained cutoff point of 1.1 g/L *(17)*. Blood samples from 183 nondiabetic subjects were consecutively selected, after excluding those on lipid-lowering or any other drugs or situations known to affect lipoprotein metabolism. All subjects gave written informed consent.

Patients were stratified according to the LDLc concentrations obtained by the different methods, following the cutoff points recommended by the National Cholesterol Education Program (NCEP) to define risk categories: ≤2.59, 2.60–3.36, 3.37–4.13, 4.14–4.91, and >4.91 mmol/L. They were also classified according to the LDLc concentration above which pharmacological intervention is recommended (3.36 mmol/L for patients without and 2.59 mmol/L for patients with previous cardiovascular events) *(1)*.

Total cholesterol and triglycerides were measured by enzymatic methods *(18, 19)* (CHOP-PAP and GPO-PAP, respectively; Roche Diagnostics). Total cholesterol was calibrated using calibration material from Roche, with a value assigned by the modified Abell-Kendall method recommended by the CDC. HDLc was measured by both precipitation, using phosphotungstate/$MgCl_2$, and by a direct method (both from Roche Diagnostics) *(20)*. External quality-control programs rendered mean inaccuracies and imprecisions lower than ± 2.6% and 2.0%, respectively, for all of the methods described above. apoB was measured by immunoturbidimetry (Roche Diagnostics), standardized against WHO/IFCC SP3-07 *(21)*. Between-batch imprecisions and inaccuracies of the apoB assay, assessed by commercial controls (Precinorm-L and Precipath-L; Roche Diagnostics) were 4.4% and 2.6% and −0.6% and −2.6%, respectively.

LDLc was calculated by the Friedewald formula using the HDLc value obtained by precipitation (LDLc-Fp) and by the direct method (LDLc-Fd), and was also determined by a modified β-quantification method (LDLc-R) separating VLDL at d <1.006 kg/L by ultracentrifugation (at 105 000g for 18 h at 4 °C) and measuring HDLc after precipitation in the infranatant with phosphotungstate/$MgCl_2$ *(2)*.

A multiple regression analysis was performed to identify the best predictors of LDLc-R in the control population, and an equation that includes apoB (in g/L), triglycerides, and total cholesterol (both in mmol/L) was obtained [LDLc-apoB = (0.385 × total cholesterol) + (2.010 × apoB) − (0.342 × triglycerides); r = 0.994; P <0.001] and used to calculate LDLc in the diabetic population. Bias against LDLc-R was evaluated for all three equations at ± 4%, as recommended by the NCEP *(5)*, and at ± 10%, which is used frequently in clinical settings.

Table 1. Bias of LDLc estimation by the three equations in all type 2 diabetic patients, compared with the recommended method, and therapeutic approach derived from their application.

	Percentage of results with bias			Therapeutic approach		
	Less than ±4%	Greater than −4%	Greater than 4%	Correct approach, % (kappa index)	Necessary ttmt[a] omitted, %	Unnecessary ttmt given, %
LDLc-FP	27.4	68.4	4.2	87.4 (0.706)	11.6	1.0
LDLc-Fd	31.6	64.2	4.2	89.5 (0.749)	9.5	1.0
LDLc-apoB	46.3	21.1	32.6	93.7 (0.818)	0	6.2

[a] ttmt, treatment.

SPSS 8.0 for Windows (SPSS Inc) was used for statistical analysis. Differences between groups were analyzed by the Student or Mann–Whitney tests, and a paired *t*-test was used to compare means within a group. $P < 0.05$ was considered significant. Concordance between the different equations and LDLc-R in the diagnosis and treatment of patients was assessed by kappa (k) index (0.21–0.40, 0.41–0.60, 0.61–0.80, and 0.81–1.0, which show fair, moderate, good, and very good concordance, respectively) (22).

Ninety-one of 95 patients had triglyceride concentrations <4.6 mmol/L, and apoB was increased in 26 of the 27 hypercholesterolemic and in 35 of the 68 normocholesterolemic subjects. The results obtained by LDLc-apoB (3.78 ± 0.77 mmol/L) were equivalent to those obtained by LDLc-R (3.76 ± 0.86 mmol/L), whereas results obtained by LDLc-Fp (3.49 ± 0.84 mmol/L) and LDLc-Fd (3.52 ± 0.84 mmol/L) were lower ($P < 0.0005$; see Table 1). Table 1 compares the bias of the equations, and Fig. 1 shows the accuracy of patient classification into risk categories according to LDLc estimation by the different equations compared with LDLc-R. The best concordance was obtained by LDLc-apoB, the only equation to achieve a k >0.6. According to their LDLc-R concentrations, 44 of the 66 patients who had not and 27 of the 29 who had suffered a cardiovascular event were candidates for drug therapy. Table 1 shows the correct and incorrect therapeu-
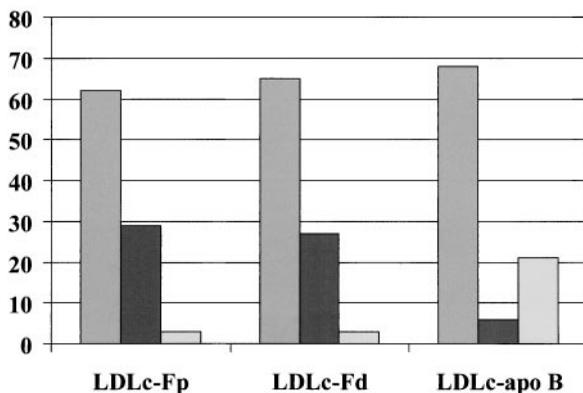

Fig. 1. Classification of patients into NCEP risk categories according to the three equations assessed.

Kappa values: 0.554, 0.585, and 0.616 for LDLc-Fp, LDLc-Fd, and LDLc-apoB, respectively. ■, correct; ■, underestimated; ▨, overestimated. Results are given as percentages.

tic decisions that resulted from the application of international guidelines (1) to the different LDLc estimations.

Cardiovascular disease is highly prevalent and is the principal cause of death in diabetic subjects (23, 24). This high risk can to a certain extent be explained by the lipid abnormalities found in this population (10). LDLc, albeit often normal or only slightly increased in type 2 diabetic patients, is the main marker used to assess cardiovascular risk and make therapeutic decisions (1, 2). To our knowledge, this is the first time that the impact of the Friedewald equation on therapeutic approach has been evaluated after individual patient assessment. The impact of LDLc estimation on patient classification has previously been evaluated using NCEP risk categories alone, without taking patients' cardiovascular disease histories into account. However, we have tried to stress the importance of taking patients, not just their numbers, into consideration. Our results show that a new equation that includes apoB allows a more accurate estimation of LDLc than the Friedewald equation, with consequences on patient risk assessment and treatment.

In agreement with most of the studies performed on type 2 diabetic subjects (12, 13), the LDLc concentrations obtained by both forms of the Friedewald equation were significantly lower than LDLc-R. Furthermore, the present results suggest that direct HDLc measurements not only are equivalent to those based on precipitation (25), as recommended by the NCEP (14), but can also somewhat improve LDLc calculation (Table 1). LDLc-apoB achieved a lower bias; its mean value was indistinguishable from LDLc-R. Most patients had triglyceride concentrations <4.6 mmol/L, which means that the advantage of the new formula is not attributable to inappropriate application of the Friedewald equation. Diabetic dyslipidemia includes lipoprotein abnormalities, which may cause underestimation of LDLc by the Friedewald formula. On the other hand, LDL particles contain >90% of total apoB, and each LDL particle carries one apoB molecule (26). Thus, a good estimation of LDLc should be expected when total triglycerides, total cholesterol, and apoB are used for its calculation.

Albeit not inexpensive, the apoB assay also adds important clinical information for the evaluation of cardiovascular risk because increased apoB not only is associated with cardiovascular disease (27, 28), but frequently is found in normocholesterolemic type 2 diabetic patients (17). The classification of patients into risk categories is

used to design therapeutic strategies. Almost 75% of the patients we assessed were eligible for pharmacological treatment. Thus, the need for an accurate estimation of LDLc to determine therapeutic intervention is evident. Nevertheless, unlike in the present study, this point has, to our knowledge, previously been assessed based on lipid concentrations alone. Both forms of the Friedewald equations underestimated cardiovascular risk and the need for drug intervention, which would be omitted inappropriately in ~10% vs in no cases according to LDL-apoB.

In conclusion, equations used to calculate LDLc concentrations in type 2 diabetes are far from ideal. The inclusion of apoB in the estimation decreases its bias and allows identification of additional patients at risk. Until direct LDLc methods have been thoroughly assessed, we may recommend that the proposed formula be used for LDLc estimation in type 2 diabetic patients.

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Presence of Fetal RNA in Maternal Plasma, *Leo L.M. Poon,[1] Tse N. Leung,[2] Tze K. Lau,[2] and Y.M. Dennis Lo[1*]* (Departments of [1] Chemical Pathology and [2] Obstetrics and Gynecology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong SAR; * address correspondence to this author at: Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Room 38023, 1/F Clinical Sciences Building, 30-32 Ngan Shing Street, Shatin, New Territories, Hong Kong SAR; fax 852-2194-6171, e-mail loym@cuhk.edu.hk)

The discovery of fetal DNA in maternal plasma *(1)* has opened up a new horizon on prenatal molecular diagnosis. Many groups have since shown that fetal genetic traits, such as RhD status and inherited genetic diseases, can be determined from fetal DNA in maternal plasma *(2–5)*. However, it is not known whether fetal RNA is also present in maternal plasma. Here, using a two-step reverse transcription (RT)-PCR assay, we demonstrate the presence of fetal-derived, male-specific mRNA in plasma of pregnant women carrying male fetuses.

Pregnant women attending the Prenatal Diagnosis Unit at the Department of Obstetrics and Gynecology, Prince of Wales Hospital, Hong Kong were recruited with informed