

Comparison of a Homogeneous Assay With a Precipitation Method for the Measurement of HDL Cholesterol in Diabetic Patients

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OBJECTIVE — To compare direct-measured HDL cholesterol with HDL cholesterol measured by a precipitation method.

RESEARCH DESIGN AND METHODS — We compared a homogeneous assay for direct HDL cholesterol analysis with the phosphotungstic acid magnesium chloride precipitation method in 55 type 1 diabetic patients, 70 type 2 diabetic patients, and 82 nondiabetic normal control subjects with plasma triglyceride levels <4.6 mmol/l. The cholesterol content of HDL determined by the direct assay was overall 0.1 mmol/l higher in all three groups than HDL cholesterol measured after precipitation, but the two methods were closely correlated ($r^2 = 0.98$, $P < 0.001$).

RESULTS — HbA_{1c}, blood glucose, serum albumin, serum bilirubin, or triglyceride did not influence the differences of the two HDL cholesterol measurements. Because we have previously shown HDL cholesterol isolated by phosphotungstic acid precipitation to be lower than that by ultracentrifugation, the positive bias found in this study was expected. It seems that the direct HDL cholesterol assay reacts with apolipoprotein (apo) B-containing lipoproteins in the fraction with a density of >1.063; these apo B-containing lipoproteins are suggested to be coprecipitated with the phosphotungstic acid method. We also measured LDL cholesterol directly by a LDL cholesterol plus method and found no significant differences between this method and LDL cholesterol calculated from Friedewald's formula.

CONCLUSIONS — Direct homogeneous assay for HDL cholesterol determination in diabetic patients seems not to exhibit a negative bias, in contrast to the precipitation method, when compared with the ultracentrifugation method. In addition, the direct assay saves time and is not influenced by type of diabetes or degree of metabolic control.

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Clinical atherosclerotic disease is positively associated with total and LDL cholesterol and inversely associated with HDL cholesterol (1–4). Diabetic patients have an increased risk of atherosclerosis, and diabetic dyslipidemia with elevated LDL cholesterol and low HDL cholesterol is common, especially in type

2 diabetic patients (5–7). In diabetic patients, the degree of glycemic control and presence of late complications seem to be associated with the level of LDL and HDL cholesterol. This situation has prompted a substantial demand for accurate LDL and HDL cholesterol quantitations.

Previous studies have questioned the

routine use of calculated LDL cholesterol by Friedewald's formula (8) in patients with type 2 diabetes, and we demonstrated earlier systematic differences in the HDL and LDL cholesterol determined by the phosphotungstic acid/MgCl₂ precipitation method compared with the ultracentrifugation method in type 1 diabetic patients (9).

In this study, HDL and LDL cholesterol were quantitated by direct homogeneous assays in type 1 and type 2 diabetic patients. The results were compared with those obtained by the phosphotungstic acid/MgCl₂ precipitation method for HDL cholesterol determination, and LDL cholesterol was calculated with Friedewald's formula.

RESEARCH DESIGN AND METHODS

Patients

We recruited 57 type 1 diabetic patients and 77 type 2 diabetic patients from the outpatient clinic at Steno Diabetes Center. Two type 1 diabetic patients and seven type 2 diabetic patients were excluded because they had a fasting triglyceride level of >4.5 mmol/l. The current age of the remaining 55 type 1 diabetic patients was between 21 and 74 years (mean 51) and for the type 2 diabetic patients between 24 and 83 years (mean 59). A total of 82 nondiabetic patients recruited from the Department of Cardiology at Copenhagen County Hospital, Gentofte, served as control subjects (mean age 50 years, range 31–61). All patients were studied after a 10-h fast and before injecting insulin or taking medicine in the morning. A cannula was inserted in the antecubital vein, and blood was collected in gel-coated glass tubes. The blood samples were kept at room temperature for 30 min before serum was obtained by centrifugation at 2,000g for 10 min.

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Abbreviations: apo, apolipoprotein.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

Table 1—HDL and LDL cholesterol in diabetic patients and control subjects

	n	HDL cholesterol		LDL cholesterol	
		Directly measured (mmol/l)	Measured after precipitation (mmol/l)	Directly measured (mmol/l)	Calculated (mmol/l)
Control subjects	82	1.58 ± 0.46	1.47 ± 0.46	3.30 ± 0.95	3.28 ± 0.93
Type 1 diabetic patients	55	1.69 ± 0.42	1.58 ± 0.41	3.49 ± 0.85	3.44 ± 0.89
Type 2 diabetic patients	70	1.29 ± 0.34	1.18 ± 0.34	3.61 ± 1.14	3.58 ± 1.11

Data are means ± SD. HDL cholesterol was measured directly or after precipitation of apo B-containing lipoproteins, and LDL cholesterol was measured directly or calculated using Friedewald's formula.

Cholesterol and triglyceride measurement

Cholesterol and triglyceride were determined enzymatically with the cholesterol oxidase/*p*-aminophenazone method and the glycerophosphate oxidase/*p*-aminophenazone method, respectively (Boehringer Mannheim, Mannheim, Germany)

Homogeneous HDL cholesterol assay

The reagents for the homogeneous HDL cholesterol assay were obtained from Boehringer Mannheim. In short, the method uses polyethylene glycol (PEG)-modified enzymes and sulfated α -cyclodextrin and dextran sulfate. PEG-modified cholesterol esterase and cholesterol oxidase enzymes show selective catalytic activities toward lipoprotein fractions, with the reactivity increasing in the following order: LDL < VLDL < HDL. In the pres-

ence of magnesium ions, sulfated α -cyclodextrin reduces the reactivity of cholesterol, especially in chylomicrons and VLDLs, without the need for precipitation of lipoprotein aggregates.

HDL cholesterol determination by phosphotungstic acid and magnesium chloride precipitation

The precipitating reagent consisted of 0.55 mmol/l phosphotungstic acid and 25 mmol/l magnesium chloride. Plasma (0.2 ml) was mixed in a tube containing the precipitation reagent (0.5 ml) and, after 10 min at room temperature, was centrifuged for 10 min at 2,000g. The clear supernatant was separated and stored in capped glass tubes for a maximum of 2 days at 4°C before the cholesterol content was determined by the cholesterol oxi-

dase/*p*-aminophenazone method, as described above (10,11).

Calculated LDL cholesterol

Calculated LDL cholesterol was derived from the following (8):

$$\text{LDL cholesterol} = \text{total cholesterol} - (\text{HDL cholesterol} + 0.47 \text{ triglycerides})$$

HDL cholesterol derived from the precipitation method was used in the equation.

LDL cholesterol plus method for direct LDL cholesterol determination

The reagents for the LDL cholesterol plus method for direct LDL cholesterol determination were obtained from Boehringer Mannheim. In short, the method uses a nonionic surfactant to solubilize LDL cholesterol, increasing the relative reac-

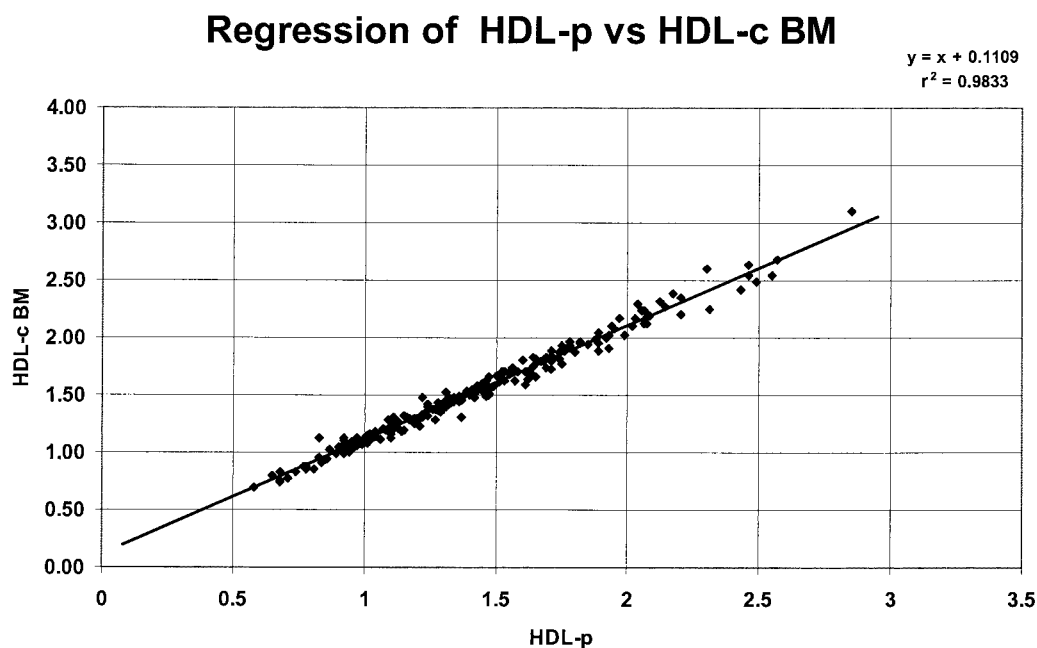
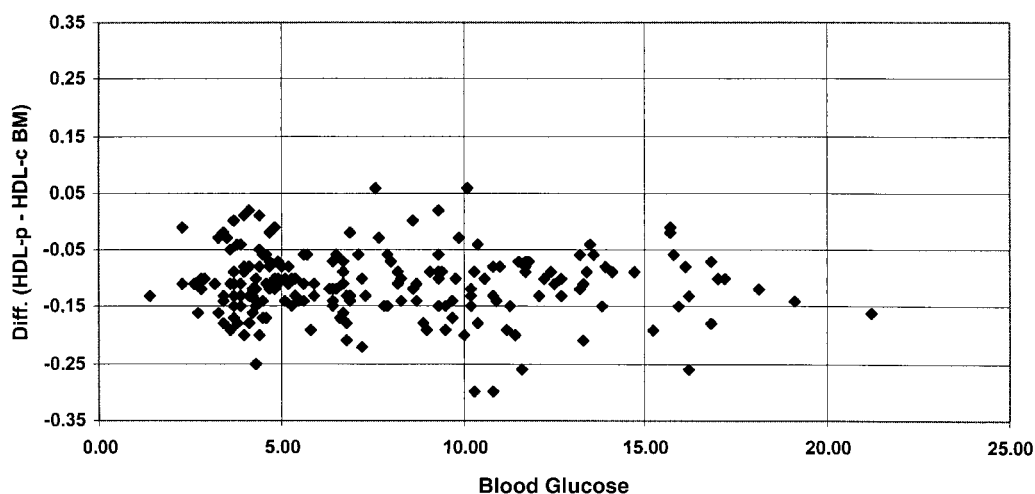


Figure 1—Correlation between HDL cholesterol measured directly (HDL-c BM) and HDL cholesterol measured after precipitation of apo B-containing lipoproteins (HDL-p) (n = 207).

Difference between HDL-p and HDL-c BM vs Glucose



Difference between HDL-p and HDL-c BM vs HbA1c

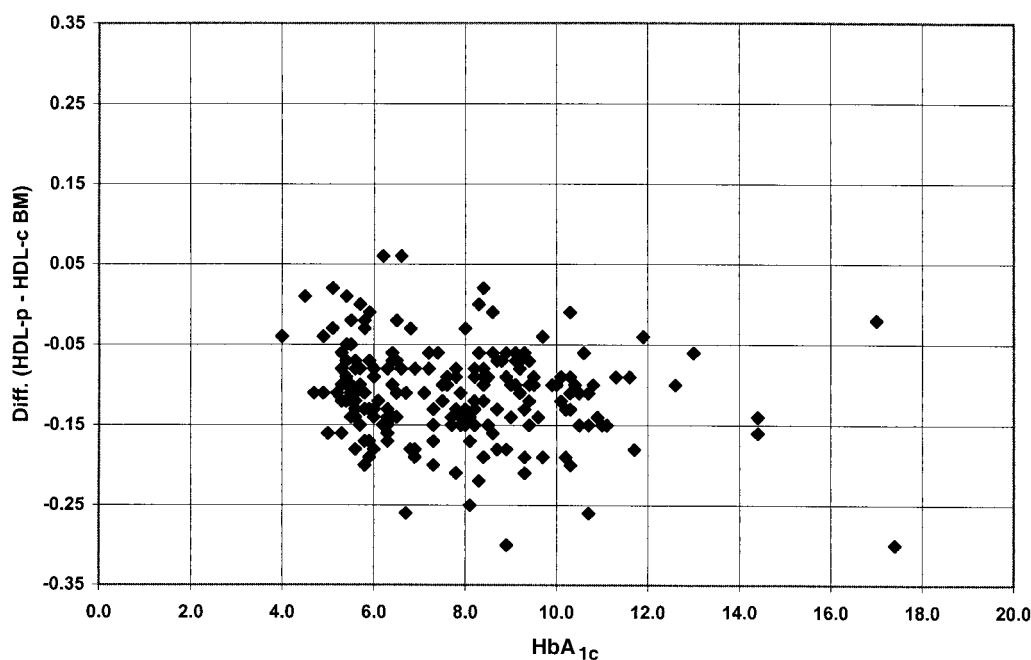


Figure 2—The effect of increased blood glucose and increased HbA_{1c} on the bias of the direct homogeneous assay for HDL cholesterol determination. Bias is the difference between HDL cholesterol measured after precipitation of apo B-containing lipoproteins (HDL-p) minus directly measured HDL cholesterol (HDL-c BM) (n = 207).

tivity of cholesterol for enzymatic determination by the cholesterol esterase-cholesterol oxidase coupling reaction in this lipoprotein fraction. In addition, a sugar compound and Mg²⁺ reduces the enzymatic reaction of the cholesterol

measurement in VLDLs and chylomicrons.

Serum albumin was measured on a Hitachi 912 with a calorimetric assay. Blood glucose was measured using a One Touch II (LifeScan). Bilirubin was mea-

sured on the Hitachi 912 with the use of 2,5-dichlorophenyldiazonium tetrafluoroborate as the diazonium salt. The absorbance was monitored at 570 nm. HbA_{1c} was measured by a high-performance liquid chromatography method (Bio-Rad

Variant from Bio-Rad Diagnostics Group, Hercules, CA)

Statistical methods

All values are reported as means \pm SD. Comparisons between and within patient groups were performed using paired and unpaired Student's *t* tests. Systematic error, including the constant and the proportional error, was derived from the linear regression equation $y = bx + a$, where *b* is the slope of the regression equation and represents the proportional error, *x* is HDL cholesterol determined after precipitation, and *a* is the *y*-axis intercept and represents the constant error. Comparisons between the methods were analyzed using the Bland-Altman difference plots. Difference plots were performed between the precipitation HDL cholesterol method and the homogeneous HDL cholesterol method. Bias of the two methods was plotted against serum bilirubin, serum albumin, HbA_{1c}, and blood glucose.

RESULTS — Serum cholesterol levels (mean \pm SD) in type 1 and type 2 diabetic patients and normal control subjects were 5.7 ± 1.0 , 5.7 ± 1.3 , and 5.4 ± 1.1 mmol/l, respectively. Measured and calculated HDL and LDL cholesterol in the three groups are shown in Table 1.

Direct measured HDL cholesterol was closely correlated to HDL cholesterol measured after precipitation ($r^2 = 0.98$; $P < 0.001$) (Fig. 1), but in all patient groups, direct measured HDL cholesterol was 0.1 mmol/l higher than HDL cholesterol measured after precipitation.

In Fig. 2, the bias of HDL cholesterol determined after precipitation minus the direct HDL cholesterol assay is plotted against HbA_{1c} and blood glucose, respectively. HbA_{1c}, blood glucose, serum albumin, or serum bilirubin (the latter two not shown in figures) did not influence the differences of the two HDL cholesterol measurements. No significant differences were found between direct measured and calculated LDL cholesterol (Table 1). No systematic bias was found among blood glucose, HbA_{1c}, serum albumin, serum bilirubin triglyceride, HDL cholesterol, and the difference of the two LDL cholesterol measurement methods.

CONCLUSIONS — In this study, we compared, in diabetic patients, a homogeneous assay for direct automated HDL

cholesterol analysis with the phosphotungstic acid magnesium chloride precipitation method. The homogeneous assay exhibited a positive bias; results for HDL cholesterol were overall 0.1 mmol/l higher than the values determined after precipitation. We and others (9,12) have previously shown HDL cholesterol isolated by phosphotungstic acid magnesium chloride to be lower than that by ultracentrifugation, suggesting that the fraction with a density of >1.063 includes some apolipoprotein (apo) B-containing lipoproteins. It seems that the homogeneous direct HDL cholesterol assay reacts with these lipoproteins, suggesting that the HDL cholesterol results obtained by this method are more in accordance with results obtained after ultracentrifugation than after precipitation.

As expected, we found lower HDL cholesterol concentrations in type 2 diabetic patients than in type 1 diabetic patients and normal control subjects. However, we could not demonstrate any bias from diabetes on the homogeneous assay minus the precipitation assay. In accordance with this, the long-term metabolic control, reflected by HbA_{1c}, and blood glucose did not influence the difference between the two HDL cholesterol measurement methods.

Although a bias of 0.1 mmol/l between the two HDL cholesterol measurements seems small, the clinical implication of the difference cannot be neglected. According to the recent guidelines from the American Diabetes Association, a cutoff point of HDL cholesterol below 1.0 mmol/l supports dietary or drug therapy for diabetic dyslipidemia (13). Only 14 patients had HDL cholesterol below 1.0 mmol/l when the homogeneous assay was used, whereas this was the case for 34 individuals when the precipitation method was used. In the subgroups, the figures were as follows: normal control subjects, 4 vs. 12; type 1 diabetic patients, 0 vs. 2; and type 2 diabetic patients, 10 vs. 22, respectively.

In vitro experiments have shown that the contents of sucrose and protein in serum influence the quantification of HDL cholesterol (10). Our study demonstrates that, within a wide but physiological range of glucose, albumin (32–49 g/l), and bilirubin ($<36 \mu\text{mol/l}$), the quantification of HDL cholesterol is not significantly affected. Serum triglyceride in the

range <4.6 mmol/l also did not influence the bias of the two methods.

In patients with diabetic dyslipidemia with moderately elevated serum cholesterol, measurement of HDL cholesterol will often be helpful in determining whether cholesterol-lowering therapy should be initiated. We previously demonstrated that the precipitation method for HDL cholesterol correlated well with the ultracentrifugation method but that the precipitation method exhibited a negative bias for HDL cholesterol. This negative bias seems not to be present for the homogeneous direct HDL cholesterol assay, thereby reducing the risk of misclassification of patients by the use of this method. In addition, the direct assay saves time, which is of great importance in diabetic patient groups where repetitive measurement of serum lipids during follow-up for many years is necessary.

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