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NOTE

Biological Variation of Serum Lipids and Lipoproteins in Patients with Clinically Well Controlled Non Insulin Dependent Diabetes Mellitus

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Abstract. To investigate how the visit-to-visit variation in serum lipids measurements affects the decision making concerning treatment according to the National Cholesterol Education Program (NCEP) guidelines in patients with clinically well controlled non-insulin-dependent diabetes mellitus (NIDDM) we have measured the biological variation (CV_b) in serum total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) in 26 patients with NIDDM. We found the CV_b as follows: TC, 5.1%; TG, 17.0%; HDL-C 4.4% and LDL-C, 8.3%. Confidence intervals (95%) were determined with total intra-individual variance values around the NCEP cut-off points to evaluate how well one, two and three lipid measurements provided reliable risk classification. A single TC measurements <177 mg/dL or >263 mg/dL allowed confident classification as "desirable" or "high risk" respectively. For LDL-C, one measurement was accurate only at below 106.3 mg/dL or above 183.7 mg/dL. The average of three measurements contracted these limits to <186.7 mg/dL and >253.3 mg/dL for TC, and <116.3 mg/dL and >173.7 mg/dL for LDL-C. For HDL-C also, multiple measurements improved risk assignment in a similar fashion. There were no values which allowed assignment to the "borderline high" category with one TC measurement and with one and two LDL-C measurements. The mean of three TC and three LDL-C measurements allowed assignment to the "borderline high" category, if between 213.3 and 226.7 mg/dL for TC, 143.7 and 146.3 mg/dL for LDL-C. Seven patients (26.9%) in this risk group based on the mean of two LDL-C estimates could be placed into a different category when the mean of three estimates was taken, even though the first two LDL-C test results did not differ by more than 30 mg/dL. Our results suggest that repeated lipid measurement is important especially for the "borderline-high" risk group because big variations existed in some patients, and further that TC is the most reliable quantity.

Key words: Intra-individual variation, Diabetes mellitus, Cholesterol, Triglyceride, Lipoproteins
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EPIDEMIOLOGICAL studies indicate that diabetes mellitus (DM) is associated with a three to fourfold increase in risk for coronary heart disease (CHD) [1–3]. Plasma lipid and lipoprotein abnormalities are commonly observed in many diabetic individuals with non-insulin-dependent diabetes mellitus (NIDDM) [4]. The United States National Cholesterol Education Program (NCEP) Adult Treatment Panel II recommends the measurement of serum total cholesterol and HDL-C in all adults 20 years of age and older [5]. To decrease the incidence of coronary events, it is recommended that people must be primarily categorised as high or borderline-high risk according to the LDL-C lev-

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el and CHD risk factor status and then be treated as previously described in the first NCEP report [6]. But the classification of people into high, borderline and low-risk categories is complicated because considerable differences in lipids and lipoproteins exist among individuals [7–12]. These differences may originate in both variations in the accuracy and precision of the laboratory methods used and also normal biological fluctuations occurring within an individual with time. The use of a single measurement to determine the risk category can result in misclassification because of the unreliability of the total cholesterol measurement resulting from both analytical imprecision and biological variation [13] and may cause financial and psychological problems [14, 15]. Now attention must be concentrated on determining and reducing the magnitude of biological variation [16].

Studies which examine the normal biological variation in lipids and lipoproteins have generally been conducted on normal subjects [7–12], but the biological component must be studied for each disease, because the disease itself and the drugs used for its treatment, may influence the extent of intraindividual variation [17]. To our knowledge, this study is the first to examine the effect of biological variation in TC, LDL-C, HDL-C and triglyceride on the cardiovascular risk assessment according to the NCEP Adult Treatment Panel II guidelines in patients with clinically well-controlled NIDDM. Fasting lipid and lipoprotein cholesterol levels were measured in diabetic patients on three separate occasions and then these single and multiple measurements were used to test the accuracy of classifications determined by the NCEP.

Materials and Methods

Patients

All subjects with NIDDM were volunteers and selected from among outpatients patients who were on follow-up in the endocrinology department. None of the patients had hypothyroidism, nephrotic syndrome, hepatic or renal disease or received drugs known to affect lipid metabolism. The inclusion criteria were that the subjects be non-smokers, their HbA_1 levels be under 8.0%, be treated by diet and oral hypoglycemic agents, have a body mass index <32 kg/m^2 and have normal blood pressure (140–90 mmHg). The protocol started in 26 patients (mean age 56 ± 11 years, 19 women and 7 men) and each patient was tested weekly on three consecutive Mondays. To minimise pre-analytical variation as much as possible, they were asked to fast for 12–14 h before each visit and the same phlebotomist collected the specimens in plain vacutainer tubes at approximately the same time of day (between 0800 and 0930 h) from seated patients for at least 10 min. Sera were separated by centrifugation (3000 g, 15 min) and aliquoted into two tubes.

Medical history and physical examination results, medication, and general health condition were recorded on the medical chart at the first visit. During this study period they were instructed to maintain their diabetic diets, weight, medication and life style, and alcohol drinking was also not allowed. The recommended caloric levels were tailored to individual needs based on an individual's desired weight and patient's activity patterns, since caloric needs vary with the patient's age, sex and activity level. The distribution of the daily caloric intake into carbohydrates, lipids and proteins was 55–60%, 30–35% and 15%, respectively. Polyunsaturated, saturated and monounsaturated lipids provided 6–8%, <10% and 30-(saturated + polyunsaturated fat)% of total energy, respectively. Daily cholesterol intake was restricted to <300 mg. Daily protein intake was 0.85 g/kg body weight. It was recommended that carbohydrates be predominantly complex and high in soluble dietary fibers; food with a low glycemic index was preferred.

The known diabetes duration of the patients was 4.9 ± 2.3 years. Two patients had only noproliferative (background) retinopathy, 2 patients had stage 1 diabetic neuropathy and 2 patients had both microalbuminuria and nonproliferative retinopathy.

Analytical procedures

Total serum cholesterol and triglycerides were measured enzymatically in a Beckman Cx-5 analyzer with Boehringer-Mannheim reagents (Mannheim, Germany). HDL-C was determined by measuring cholesterol in the supernatant liquid after precipitation of apolipoprotein B-containing lipoprotein particles with phosphotungstic acid and magnesium ions (as for cholesterol plus, Reagent Set HDL-C Precipitant, Boehringer Mannheim

GmbH, Mannheim, Germany). LDL-C levels were calculated by means of the Friedewald equation [18]: [LDL-C] = [TC] − [HDL-C] − [TG]/5, where all concentrations were expressed in mg/dL and no serum TG level exceeded 400 mg/dL limit. To minimise analytical variation, all specimens from each individual were assayed in the same batch, with the same lots of reagent, standards and quality control material. All analyses were performed by a single analyst and each specimen was assayed in duplicate. We used the average of two measurements for statistical procedures.

We determined the coefficients of analytical variation (CV_a), from analyses of several serum control pools running concurrently with the study group. For total cholesterol and triglycerides we used two lyophilized pools obtained commercially (Precinorm U, Ch-B./Lot: 172006 and Precipath U, Ch.-B./Lot: 177479 Boehringer Mannheim, Mannheim Germany), and for HDL-cholesterol we used Stanbio HDL-C standard solution. For each pool we determined the mean and standard deviation (SD) and then calculated the CV_a with the following formula: $CV_a = (SD/_{mean}) \times 100$. Because the CV_a values for different quality-control sera were similar, we used the average CV_a for the control serums. The CV_a for LDL-C was calculated from the CV for TC, HDL-C and triglycerides. The CV_a were 2.1%, 5.6%, 3.1% and 3.5% for TC, HDL-C, triglycerides and LDL-C respectively.

Statistical methods

For each patient we calculated the mean, SD and variation in the lipid values for the three visits by means of a commercially available statistics program. From these data, the mean and SD for the entire population were calculated. The total variation in the measurements for three specimens from one person is composed of the biological variation and the analytical variation. The biological variations for cholesterol, triglycerides, HDL-C and LDL-C were calculated by means of the following formula:

$$(\text{Biological CV})^2 = (\text{Total CV})^2 - (\text{Analytic CV})^2$$

Dividing intraindividual SD by the square root of 2 and 3 provided the values for the average intraindividual standard error based on two and three observations, respectively. One-tailed 95% confidence intervals (CI_s) were determined by multiplying the values for standard error by 1.96 and adding the product to or subtracting the product from the limits recommended by the Adult Treatment Panel II (200 and 240 mg/dL for TC, 130 and 160 mg/dL for LDL-cholesterol and 35 mg/dL for HDL-C) to identify the uncertain region in classification. TC, HDL-C and LDL-C values were classified according to Adult Treatment Panel II guidelines. Misclassifications were identified by comparing individual measurements with the classification of the mean.

Results

Table 1 displays the overall means (± SD) lipid and lipoprotein cholesterol concentrations in the diabetic patients. Figures 1 and 2 represent the mean and nonparametric ranges of total cholesterol and calculated LDL-C measurements for each patient. A relatively wide and narrow range of variation was observed in some patients, for example patients 14 and 24 and patients 8 and 26 for TC; patients 9 and 17 and patients 4 and 11 for LDL-C, respectively. When a comparison of a single observation *vs.* the sample mean for the same patients was done, 9 of 78 (11.5%) observations for TC, 19 of 78 (24.3%) observations for LDL-C, 7 of 78 (8.9%) observations for HDL-C were but into a different category from their associated sample means.

To assess the impact of intraindividual variation on the classification of diabetic patients' lipid levels, we constructed 95% CI_s to determine whether observed values could be confidently placed within risk groups. We did this analysis for a single measurement, for the average of two measurements, and for the average of three measurements. The risk classifications we have used are those of the NCEP Adult Treatment Panel II guidelines: for TC <200 mg/dL (desirable), 200 to 239 mg/dL (borderline-high) and ≥240 mg/dL (high); and LDL-C <130 mg/dL (desirable), 130 to 159 (borderline-high) and ≥160 mg/dL (high); and for HDL-C <35 mg/dL (low) and ≥35 mg/dL (desirable).

In Table 2 we show the 95% CI_s for TC, LDL-C and HDL-C classification when 1, 2 and 3 measurements were made. These values were based on the individual SD_s and standard error medi-

Table 1. Means, SDs and SEMs for lipid and lipoprotein concentrations in diabetic patients

Variables	Mean ± SD	SEM, 1 Measurements	SEM, 2 Measurements	SEM, 3 Measurements
TC (mg/dL)	204 ± 39	11.75	8.31	6.78
TG (mg/dL)	145 ± 72	28.61	20.22	16.51
HDL-C (mg/dL)	40 ± 9.3	2.43	1.72	1.40
LDL-C (mg/dL)	131 ± 48.5	12.10	8.55	6.98

TC, Total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

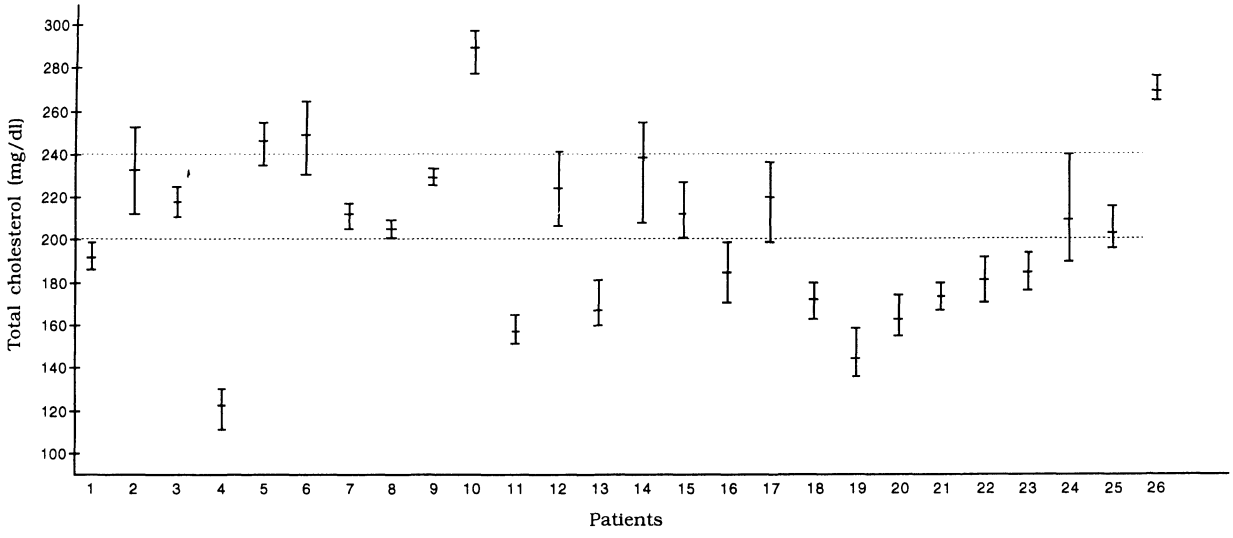

Fig. 1. Range of total cholesterol concentrations for each patient. The "tic" marks signify the lowest, the highest and the mean of three measurements.

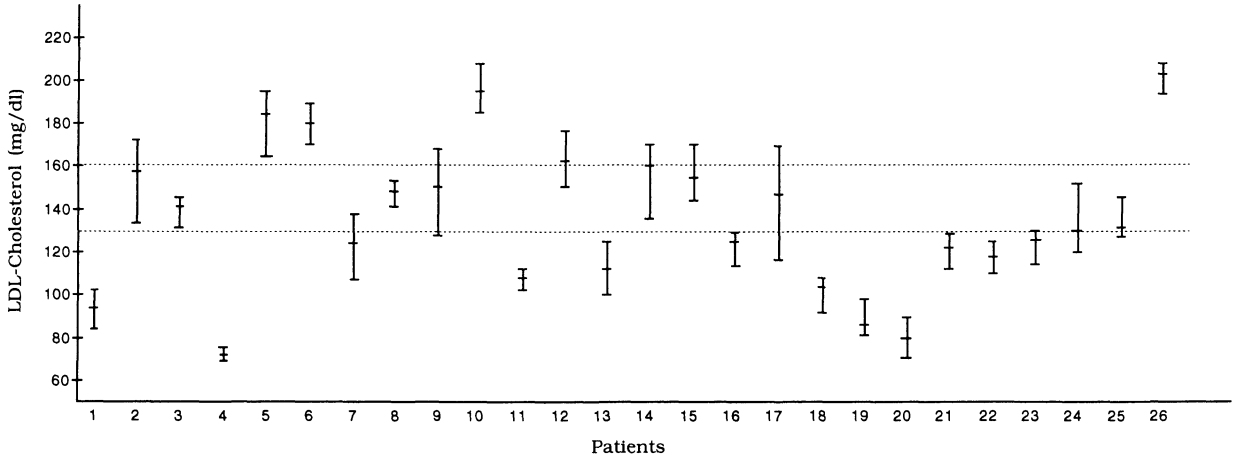

Fig. 2. Range of LDL-cholesterol concentrations for each patient. The "tic" marks signify as in Fig. 1.

Table 2. Confidence intervals for accurate classifications of total cholesterol, LDL-cholesterol and HDL-cholesterol according to the National Cholesterol Education Program criteria for 1, 2, and 3 measurements

No. of lipid Measurements	Total cholesterol			LDL cholesterol			HDL cholesterol	
	Desirable (<200)	Borderline high (200–239)	High (≥240)	Desirable (<130)	Borderline high (130–159)	High (≥160)	Low (<35)	Desirable (≥35)
1	< 177.0	No value*	> 263.0	< 106.3	No value*	> 183.7	< 30.2	> 39.8
Average of 2	< 183.7	216.3–223.7	> 256.3	< 113.2	No value*	> 176.8	< 31.6	> 38.4
Average of 3	< 186.7	213.3–226.7	> 253.3	< 116.3	143.7–146.3	> 173.7	< 32.3	> 37.7

All values are expressed as mg/dL. * "No Value" indicates that there is no overlap between the confidence intervals.

Table 3. Total, biological and analytical variation in cholesterol, LDL-C, HDL-C and triglycerides and effect of number of repeated specimens on CV_t

	CVb	CVa	CV_t No. of repeated specimens 1	2	3
TC	5.1	2.1	5.5	3.9	3.2
TG	17.0	3.1	17.3	1.2	9.9
LDL-C	8.3	3.5	9.0	6.4	5.2
HDL-C	4.4	4.1	6.0	4.2	3.2

ums (SEM_s) for 1, 2 and 3 measurements reported in Table 1. In our patients, a single total cholesterol measurement <177.0 mg/dL and >263.0 mg/dL would provide accurate classification with 95% confidence for desirable and high risk categories. Averaging two measurements improved the accuracy of classification for total cholesterol, but the mean of three measurements allowed a some-what better risk assignment than the average of two measurements. But in assessing the borderline-high risk range according to the levels of LDL-C, averaging three measurement established a range of only 2.6 mg/dL on either side of the recommended limits in which an observed value does not allow confident assignment of a diabetic patient to the borderline-high risk category. Seven patients (26.9%) in this range based on the mean of two measurements could be classified into the desirable or high risk range when the mean of three measurements was taken.

Table 3 presents the total, biological and analytical variation in lipid and lipoprotein quantities and the effect of multiple specimens on the coefficients of total variation (CV_t) in the determined mean value. The biological variation comprised a high fraction of the total for all quantities especially for TC, triglycerides and LDL-C. Table 3 also indicates that a great improvement in decreasing CV_t occurs when measuring additional serum specimens from the same person.

Discussion

Long term DM is associated with atherosclerotic vascular disease. The lipoprotein alterations observed in diabetic patients are partly responsible for the development of early atherosclerosis [19]. In assessing the effects of disease and medication used for its treatment on the lipoprotein profile, a knowledge of intra-individual variation is important. Determining the patients with NIDDM at high risk of having dyslipidemia depends on accurately assessing lipids. In the present study we calculated the analytical and biological components of total variation for TC, triglycerides, HDL-C and LDL-C in patients with NIDDM. Our diabetic patients treated with diet and oral sulfonylurea drugs were being strictly controlled in our endocrinology department. Therefore the CV_b of lipid and lipoproteins observed in our patients were lower than those of IDDM patients. Hölzel reported that the average CV_bs in IDDM patients were 7.3%, 8.6% and 20.9% for TC, HDL and TG but diabetic control was not be taken into consideration in this study [20]. But Bookstein *et al.* [12] reported the CV_bs of TC, HDL-C, LDL-C and TG in healthy subjects as 4.8%, 7.5%, 5.9% and 21%, respectively. They also constructed the 95% Cls for TC and LDL-C classification when 1, 2 and 3 measurements were done. Thus they have found for a single TC measurement that was below 185 mg/dL, between 215 and 225 mg/dL or greater than 255 mg/dL, one measurement made possible accurate classification. The mean of 2 or 3 TC measurements improved the accuracy of classification. Similarly a single

LDL-C measurement that was below 116 mg/dL or greater than 174 mg/dL was enough for a reliable classification but, for the borderline-high range, a single LDL-C measurement did not make possible accurate classification. But the average of three LDL-C measurements that was between 138 and 152 mg/dL made possible accurate classification for the borderline-high category. In our study, however, the values obtained by the mean of three measurements were similar to the single measurement for 95% CIs obtained by Bookstein *et al.* And when the average of two or three TC and LDL-C measurement was obtained the 95% Cls for the borderline high range were much wider than our values. We could not construct the 95% CLs for the borderline high range to provide reliable classification with the average of LDL-C measurements because of the high CV_b for LDL-C, although all of our diabetic patients were strictly metabolically controlled. It is therefore obvious that poorly controlled diabetic patients would have a much greater CV_b for lipid and lipoprotein measurements.

The CV_b of TG was increased up to 55% in some of our diabetic patients and this high variation may easily affect the CV_b of LDL-C since LDL-C is indirectly assessed according to the levels of serum TG as in the present study. Nine of 26 (35%) patients for CV_b of TG, 5 of 26 (19%) patients for HDL-C, 8 of 26 (30%) patients for CV_b of LDL-C and only 3 of 26 (12%) patients for CV_b of TC had higher values then the metaanalytic average values of 30 previously published studies related to biological variation in the concentration of serum lipids [21]. The mean CV_b values found by metaanalysis of previous published studies are $\leq$6.1%, $\leq$7.4%, $\leq$9.5% and $\leq$22.6% for TC, HDL-C, LDL-C and TG, respectively [21]. The CV_b of HDL-C was the smallest of all other quantities but its CV_a was the greatest. But all of the CV_as of lipid and lipoprotein in this study were lower than the values accepted by the NCEP laboratory standardization panel [22]. The NCEP-recommended CV_a goals are 3% for TC, 6% or HDL-C, 4% for LDL-C and 5% for TG. The mean of two HDL-C measurements may be enough for reliable classification in our patients.

The NCEP Adult Treatment Panel II recommends initial classification based on total TC and HDL-C for primary prevention of CHD. For individuals with a desirable blood cholesterol less than 200 mg/dL and HDL-C levels less than 35 mg/dL should proceed to lipoprotein analysis including LDL-C measurement. Individuals with desirable LDL-C levels (less than 130 mg/dL) in a single measurement do not need further evaluation or active medical therapy. According to these recommendations, two measurements should be performed in 6 of our patients (patients Nos. 1, 4, 16, 21, 22 and 23) with a desirable blood cholesterol and low HDL-C level. As seen in Fig. 2 all of these patients had desirable LDL-C levels on three occasions. Because our findings support the NCEP recommendations, it is unnecessary to repeat LDL-C measurements in these patients with desirable TC and LDL-C levels determined by a single measurement. And, as shown in Fig. 2, a third measurement is unnecessary if the mean of two LDL-C values exceeds 176.8 mg/dL (i.e. patients Nos. 5, 6, 10 and 26).

The amount by which the lipid and lipoprotein concentrations vary over time is clinically important for the physician determining both the patient's coronary risk related to dyslipidemia and the response to dietary and drug treatments. Because of the intraindividual variation in lipids and lipoproteins, several measurements performed at least one week intervals are essential to establish the patient's usual concentrations of these components accurately as recommended by the NCEP report [6]. As seen in Table 3, CV_t for total cholesterol was 5.5% when measured once in a single sample and decreased to 3.9% if two serial samples were measured in our patients. The NCEP recommends a third measurement within one to eight weeks, if the first two TC or LDL-C values differ by more than 30 mg/dL [6]. But, because of unconstructed 95% Cls for the borderline high category according to average of multiple LDL-C measurements, the use of TC values would be more beneficial for this category classification in diabetic patients. In population screening and in clinical practice, particularly when therapeutic interventions are being considered, analytical and biological fluctuations in serum lipid levels should be taken into account. The rise or fall in serum levels of total cholesterol or LDL-cholesterol should therefore not be automatically attributed to "patient noncompliance" or "effective intervention".

In conclusion, repeated measurement of serum lipids is important especially for establishing accurate classification of the "borderline-high" risk group because big variations existed in some NID-

DM patients. But the application of the NCEP recommendations for the other categories, by means of LDL-C values, provides reliable classification.

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