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Lipids and Lipoproteins: Interpretation of Laboratory Results and Methodologies

Open Access **Review Article**

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Abstract

The main objective of this review is to consider the criteria involved with the variables that can influence the interpretation, the quality of each patient's report and the methodological principles of use in the clinical laboratory. The analysis of the sources of variation in laboratory results is considered, mainly the pre analytical and the analytical factors. Biological variability is also considered and the variation coefficient is described. Biological variability is not measured directly, but is obtained by the difference between analytical variability (precision or reproducibility of the method) and total variability (the sum of analytical and biological variability), as shown by the equation. The analytical variables are mainly those introduced in the result resulting from the methodology employed (methodological principle, quality of reagents, manual or automated processing) and the calibration system (standards) used. The perennial use of good quality control programs and compliance with good practices for the clinical laboratory allow quantifying and monitoring analytical variability. Next comes the applications of these concepts to the lipid profile. The lipid profile is defined by the last Update of the Brazilian Guideline on Dyslipidemias and Prevention of Atherosclerosis by laboratory determinations of total cholesterol, triglycerides, HDL-cholesterol, Non HDL-cholesterol, LDL-cholesterol, calculated by Friedewald formula. Other centers include as a routine, for the diagnosis of dyslipidemias, in addition to the determinations mentioned in the lipid profile. Plasma aspect after rest at 4°C, lipoprotein A and apolipoprotein B. Determinations of all the formulae to calculate the LDL cholesterol go from the Friedewald to the Martin/Hopkins formula that take all triglycerides ranges into consideration plus the fact that total cholesterol and HDL cholesterol can be assayed in non fasting conditions. Special attention is given to the particular methodological considerations regarding triglycerides assays. Recommendations as to lipid profile measurements according to age groups are pointed as well.

Keywords: Lipids; Methodology; Cholesterol; LDL Cholesterol; HDL Cholesterol; Non HDL Cholesterol; Apoprotein A-I; Apoprotein B; Triglycerides

Abbreviations: Apo A-I: apoprotein A-I; Apo B: Apolipoprotein B; BVC: Biological Variation Coefficient; CDC: Centers for Disease Control and Prevention; HDL: High Density Lipoprotein; HDL-c: High Density Lipoprotein Cholesterol; IDL: Intermediate low-density lipoprotein; LDL: Low Density Lipoprotein; LDL-c: Low Density Lipoprotein Cholesterol; Lp(a): Lipoprotein a; NCEP: National Cholesterol Education Program; Non HDL-c: Non HDL-cholesterol; NR: Number of Replicates; NS: Number of Samples; RV: Relative Variation; TC: Total Cholesterol; TG: Triglycerides; VC: Variation Coefficient; VCa%: Coefficient for Analytical Variation; VLDL: Very Low Density Lipoprotein; VLDL-c: Very Low Density Lipoprotein Cholesterol

Review

This review will address the criteria involved with the variables that can influence the interpretation, the quality of each patient's report and the methodological principles of use in the clinical laboratory.

Choice of laboratory

Suitable companies that make use of quality control programs in their routines should be chosen, such as those offered by the Brazilian Society of Clinical Pathology (Control Lab PELM) and the Brazilian Society of Clinical Analyses (National Quality Control Program-PNCQ), all affiliated to international quality control programs.

Sources of variation in laboratory results: the correct interpretation of laboratory data should take into account the variables that can modify the result. The main sources of variation in biochemical assays can be attributed to pre-analytical and analytical events *[1]*.

Pre-analytical factors are the main responsible for the variability of the results. Sources of pre-analytical variation include the correct identification, adequacy, collection and transport of the sample, and biological variables that have gained great prominence due to their influence on the

interpretation of the results *[1,2]*.

Biological Variability

Biological factors are recognized today as the main sources of variability in biochemical results. Some biological variables, such as fasting and diet prior to collection, can be controlled. Others such as gender, age, body mass, stress and medication use are difficult to standardize. It is also very important the intrinsic individual variation, peculiar to the individual, in which the analytes show a random variation around a homeostatic point *[1,3]*.

Table 1 highlights two studies describing biological variability in lipids. Marcovina SM, et aI,*[2]* evaluated 20 healthy individuals (10 men and 10 women) using blood samples collected at an interval of two weeks and Smith et aI.,(1993), studied through meta-analysis 30 studies of the literature on biological variability and characterized the mean biological variability *[2,3]*.

Analyte	Average mg/dL	Range of Variation mg/dL	BVC % média	BVC% range of variation	BVC % meta-analysis
Cholesterol	186	134 - 245	6,7	1,7 - 11,6	6,1
HDL-c	51	20 - 74	7,5	2,2 - 13,7	7,4
LDL-c	115	76 - 185	9,2	2,0 - 15,3	9,5
Triglycerides	98	42 - 334	28,2	5,3 - 74,0	22,6
Apo B	87	57 - 126	7,4	3,0 - 11,9	
Apo A-1	142	96 - 200	7.4	0,0 - 12,6	
Lp (a) expressed in protein mass	1,5	0 - 3,0	26,7		
	4,5	> 3,0 - 6,0	21,9		
	10,6	> 6,0 - 15,0	15,7		
	24,2	> 15,0	9,0		

Table 1: Biological Variation Coefficient (BVC) for lipids and lipoproteins.

The results for the same healthy individual, obtained under standardized conditions, characterize intra individual biological variability, which is expressed in terms of standard deviation (SD) or variation coefficient (VC) (VC=(SD/mean)x100). Biological variability is not measured directly, but is obtained by the difference between analytical variability (precision or reproducibility of the method) and total variability (the sum of analytical and biological variability), as shown by the equation *[1-3]*.

$$(\text{total VC})^2 = (\text{biological VC})^2 + (\text{analytical VC})^2$$

$$\text{Biological VC} = \sqrt{(\text{total VC})^2 - (\text{analytical VC})^2}$$

The Biological Variation Coefficient (BVC) for lipids shows very expressive values, capable even of modifying the interpretation of the results. Another point that draws attention is the wide dispersion of these values among individuals, as can be exemplified in triglycerides (TG) (Table 1, BVC - amplitude of variation), finding individuals with BVC of about 5% and others with 74%, characterizing the magnitude of the challenge imposed on clinicians and laboratories in the evaluation of this parameter.

Cooper and collaborators[4] working together with a North American group that studies the interpretation of lipids, supported by the Centers for Disease Control and Prevention, USA (CDC), demonstrated that the total VC of an individual reduces when more than one determination is performed (number of samples - NS) and when the number of replicates (repeated determinations of the same sample is increased, NR). They proposed the equation below, whose expected variations are shown in Table 2:

$$\text{Total VC}^2 = \frac{\text{VC}^2 \text{ analytical}}{\text{NRxNS}} + \frac{\text{biological VC}^2}{\text{NS}}$$

where: VC = variation coefficient, NR = number of replicates, NS = number of samples.

This study refers to the practice of using at least more than one sample collection to establish a reliable diagnosis in the case of lipids. Note in Table 2 that the total coefficient of variation for TG reduces by about 50% when three determinations of this parameter are performed, when compared to an isolated determination. Increasing the number of collections to establish a diagnosis can be very useful for those patients who have values borderline between desirable and at-risk. However, it should be remembered that this procedure entails an increase in costs for the patient or the health system, and therefore should be used with common sense.

Total Coefficient of Variation (%)								
Number of samples	Total cholesterol		HDL-c		LDL-c		Triglycerides	
	1 NR	2 NR	1 NR	2 NR	1 NR	2 NR	1 NR	2 NR
1	6,8	6,4	9,5	8,5	10,3	9,9	23,1	22,9
2	4,8	4,6	6,7	6,0	7,3	7,0	16,4	16,2
3	3,9	3,7	5,5	5,0	6,0	5,7	13,4	13,2
4	3,4	3,2	4,8	4,3	5,2	5,0	11,6	11,4
5	3,0	2,9	4,3	3,9	4,6	4,4	10,4	10,2

Table 2: Effect of the number of samples and number of replicates on the total variation of mean serum lipid levels.

NR = number of replicates

Analytical variability

The analytical variables are mainly those introduced in the result resulting from the methodology employed (methodological principle, quality of reagents, manual or automated processing) and the calibration system (standards) used. The perennial use of good quality control programs and compliance with good practices for the clinical laboratory-GLP -allow quantifying and monitoring analytical variability. The NCEP (National Cholesterol Education Program) has proposed several guidelines regarding methodological processes for the determination of lipids and lipoproteins*[5]*. Table 3 gives the NCEP recommendations for the analytical variables of the main lipids quantified in the laboratory. These values are based on various studies and the "state-of-the-art" methodologies and equipment available for analysis*[6]*. The coefficient of analytical variation (VCa%) is obtained by analyzing the same sample at least 20 times on different days and characterizes the accuracy or reproducibility of the method. The bias represents the degree of accuracy of the method and quantifies how much the methodology in use differs from the reference methodology, assumed as a true value; and the total error is a

parameter that quantifies the sum of the effects of the VCa plus the deviation (total error%=%deviation+1.96.VCa).

projection angle (FPPA) is commonly used to perform a 2D assessment of the amount of knee valgus for a functional task such as the step down test (SDT)*[1,2]*. However, it is possible that there is little difference between the initial FPPA at the beginning of the task and the final FPPA measurement, but considerable knee medial and lateral deviation during the descent. The deviations may be considered indicators of dynamic knee stability due to a lack of muscle control during a functional task *[3]*. Altered movement control patterns during a SDT are risk factors for painful knee

Analyte	Total Error (%)	Deviation (%)	VCa (%)
Cholesterol	≤9	≤3	≤3
HDL-c	≤22	≤10	≤6
LDL-c	≤12	≤4	≤4
Triglycerides	≤15	≤5	≤5

Table 3: Recommendations from the national cholesterol education program for lipid and lipoprotein determinations.

Total error=% deviation+1,96. VCa
Bias=difference between the actual and the observed value
VCa=coefficient of analytical variation (precision or reproducibility of the method)

The literature recommends as acceptable the result of a biochemical parameter is affected by the sum of the analytical and biological variations to which this analyte is subjected. The last Update of the Brazilian Guideline on Dyslipidemias and Prevention of Atherosclerosis[7], referring to the previous consensus of 1993, recommends as acceptable methodological and/or biological variation the values of: up to 5% for total cholesterol (TC) (ideal≤3%), up to 10% for high density lipoprotein cholesterol (HDL-c) and up to 20% for TG, these indices being expressed as VC. For two successive determinations that differ from these percentages, the Consensus recommends the realization of a third determination, considering the average of the closest determinations for the evaluation of the parameter. Thus, a TC determination of 240 mg/dL, assuming a VC of 5%, would actually represent values with the amplitude of 216 to 264 mg/dL.

$$VC = \frac{\text{standard deviation}}{\text{Average}} \times 100$$

$$5/100 = \frac{\text{standard deviation}}{\text{Average (= 240 mg/dL)}} \rightarrow \text{standard deviation} = 12 \text{ mg/dL}$$

To ensure a statistical certainty of 95% (1 failure out of 20), the mean±2 standard deviations are used, in this example 240±24 mg/dL. Therefore any subsequent determinations whose values are within this range of variation (216-264 mg/dL) are not statistically different.

The validation of mean serum values for a patient in the determinations of TC, HDL-c, low density lipoprotein cholesterol (LDL-c) and TG, with more than one determination, can be performed by the use of the "Relative Variation" (RV) described by Cooper GM, et al. *[4]*. These authors defined RV as the difference between the highest and lowest value of the analyte under study, divided by the mean obtained with all values. Based on the values of the mean BVC obtained by meta-analysis (Table 1), the analytical variation recommended by the NCEP (Table 3), and with statistical studies, Table 4 was constructed, which contains the maximum permissible values (95th percentile) for the RV of the main lipids quantified in the laboratory.

Sample Number	Total Cholesterol	HDL-c	LDL-c	Triglycerides
2	0,19	0,27	0,29	0,67
3	0,23	0,32	0,35	0,82
4	0,25	0,35	0,38	0,90
5	0,26	0,37	0,40	0,94

Table 4: Maximum relative variation values allowed.

Source of Variation	Total Cholesterol	HDL-C	LDL-C	Triglycerides
Sample				
Absence of fasting	NV	↓	↓	↑↑
Prolonged total fasting	↑↑	↓	↑	↑
Posture: standing for:				
Lying	↓	↓↓	↓↓	↓↓
Sitting	↓	↓	↓	↓
Anticoagulants for serum	↓	↓	↓	↓
Behavior				
Diet				
Saturated fatty acids (palmitic acid)	↑	NV	↑	↑
Monounsaturated fatty acids	↓	NV	↓	↓
Polyunsaturated fatty acids	↓↓	↓	↓↓	NV
Cholesterol intake	↑	NV	↑	NV
Fish oil	NV	NV	NV	↓
Obesity	↑	↓	↑	↑↑
Smoking	↑	↓	↑	↑↑
Exercises (strenuous)	↓	↑	↓	↓
Alcohol intake	↑	↑	↓	↑↑
Clinical sources				
Myocardial infarction				
24 hours after	NV	NV	NV	NV
6 weeks after	↓	↓	↓	NV
Stroke	↓	NV	↓	NV
Antihypertensive diuretics	↑	↓	↑	↑↑
Nephrosis	↑↑	NV	↑↑	↑↑
Diabetes (insulin resistance)	↑	↓	↑↑	↑↑
Infections	↓	↓	↓	↑↑
Pregnancy (> 2nd semester)	↑	NV	↑	↑↑
Transplant				
Cyclosporine	↑↑	↓	↑↑	↑
Prednisone	↑	↓	↑	↑↑

Table 5: Sources of pre-analytical variations of lipids.

NV=Does Not Vary; ↑=minimum or moderate increase; ↑↑=moderate to high increase; ↓=minimal to moderate reduction; ↓↓=moderate to high reduction.

Method	Advantages	Disadvantages
RIA	• highly sensitive • no matrix effect • uses polyvalent/monoclonal antibodies • uses primary/secondary calibrators	• difficult to automate • requires large dilutions • moderate accuracy • uses reagent radioactive compounds with short stability
ELISA	• highly sensitive • polyvalent/monoclonal antibodies • insensitive to the matrix • uses primary/secondary calibrators	• difficult to automate • moderate accuracy • requires large dilutions
Immunonefelometry	• highly automatable • good accuracy	• potential interaction of the matrix • special equipment • specific antiserum
Immunoturbidimetry	• highly automatable • uses common laboratory equipment	• variable accuracy • potential interaction of the matrix • needs specific antiserum
Radial immunodiffusion	• minimum use of special equipment • small dilution	• slow and inaccurate • matrix interaction • variability with lipoprotein species • need for specific antiserum

Table 6: Advantages and disadvantages of the main assays for quantification of apolipoproteins.

Recommendations for the use of RV and the number of serial samples to be obtained for each individual

Calculate RV by dividing the difference between two determinations by the mean of these dosages. If the RV for the two samples exceeds 0.19 for TC, 0.27 for HDL-c, 0.29 for LDL-c, or 0.67 for TG, make another determination (third sample) to establish the mean value.

If the RV for the three samples (the difference between the highest and lowest value divided by the mean obtained by the three determinations) exceeds 0.23 for TC, 0.32 for HDL-c, 0.35 for LDL-c or 0.82 for TG, make a fourth determination if the mean analyte values require a more accurate estimate

Exemplifying the use of RV. One patient performed two triglyceride determinations 15 days apart, resulting in 220 and 390 mg/dL. Can the clinician assume for this patient the mean value of 305 mg/dL of TG and use this base horn value for their clinical decisions?

Calculating RV

1st determination = 220 mg/dL
2nd determination = 390 mg/dL
Difference (220 - 390) = 170 mg/dL
Average = (220 + 390)/2 = 305 mg/dL
RV = difference/mean = 170/305 = 0.56

The calculated RV value of 0.56 is less than the tabulated value for TG with 2 samples 0.67(Table 4), so this difference between the two determinations can be explained by the effect of the average biological and analytical variability of the individuals, and the mean value of the two determinations presents statistical consistency as a guide for diagnosis and/or treatment basis.

According to Cooper GM, et al.,*[4]*, the use of the RV is advantageous in determining the true mean of the lipid values of a sample because: 1) the calculation of the VC is unnecessary, 2) cancels the effect of the concentration on the standard deviation, 3) makes available a technique that quickly estimates the effect of biological variation using only two or four samples, and 4) reminds clinic and laboratory professionals of the potential effects of biological variations on

lipid results.

Lipid Profile

The lipid profile is defined by the last Update of the Brazilian Guideline on Dyslipidemias and Prevention of Atherosclerosis *[7]* by laboratory determinations of:

a. TC
b. TG
c. HDL-c
d. Non HDL-cholesterol (Non HDL-c)
e. LDL-c (calculated by Friedewald formula)

LDL-c=TC-HDL-c-TG/5

Friedewald formula limitations: TG ≥ 400 mg/dL

Other centers include as a routine, for the diagnosis of dyslipidemias, in addition to the determinations mentioned in the lipid profile *[8]*:

a. Plasma aspect after rest at 4°C
b. Lipoprotein a (Lp(a))
c. Apolipoprotein B (apo B)
d. Among the procedures that can be performed as additional tests are:
e. Apoprotein A-I (apo A-I)
f. Fibrinogen
g. Indices or ratios of biochemical parameters (especially in population surveys):
h. Risk index (or Castelli index) *[9]*
i. Risk I = TC/HDL-c
j. Risk II = LDL-c/HDL-c
k. ApoB/ApoA-I

Target population of the lipid profile: it is recommended that the lipid profile be performed in the populations defined below *[3,7]*:

a. Men and women 20 years of age or older
b. In the presence of atherosclerotic disease (coronary, cerebrovascular, carotid, aortic and/or its terminal branches)
c. Children and adolescents (2 to 19 years of age) when:
d. Have clinical signs of dyslipidemia.
e. Have other risk factors for atherosclerotic disease.
f. Have a family history of dyslipidemia or family history of early atherosclerotic disease in first-degree relatives. Early occurrence of atherosclerotic disease is defined in men before 55 years of age, and in women before 65 years of age.
g. Presence of acute pancreatitis, xanthotosis, obesity or other risk factors for coronary artery disease.

Being the result of the first normal lipid profile, it is advisable to repeat it every 5 years, provided that there is no appreciable variation in life habits (smoking, high-fat diet) or earlier at medical discretion.

Pre-analytical procedures: it is essential to obtain accurate and clinically discriminating results, to standardize all possible pre-analytical and analytical variables to minimize variability in results. Examples of pre-analytical variables and their implications on lipid profile are found in Table 5.

Sample collection

Patient preparation *[3,7]*

a. Fasting for 12-14 hours, usually nocturnal, free water intake.
b. Patient with diet and usual activities. Do not perform lipid determinations in patients with acute or chronic diseases (cancer, infections, among others) and in the postoperative period of major surgeries. In reversible clinical conditions it should be waited until about 3 months after recovery for lipid results to return to the usual levels for the patient.
c. Avoid drinking alcoholic beverages on the day before sample collection.
d. Stable body weight in the last 4 weeks.
e. Interpretation of the use of drugs that increase or reduce lipids such as: oral contraceptives, thyroid hormones, steroids, anti hypertensives, diuretics, hypoligemiants.

Sample

The collection should be performed with the patient seated (or lying down) who held this position for at least 5 minutes before the puncture. Do not collect samples in patients who underwent vigorous physical exercise 24 hours prior to collection.

The tourniquet should be applied for the shortest possible time (ideal<1 minute no more than 2 minutes) to avoid venous stasis.

For all lipid profile assays and apolipoprotein determinations the sample of choice is serum. Plasma obtained with EDTA can be used, being recommended for determination of lipoproteins for its stabilizing effect by 40 metals. It is important to remember that the presence of this anticoagulant introduces a reduction of approximately 3% in the determination of TC, TG and HDL-c, by the osmotic redistribution of water between cells and plasma, which should be corrected by multiplying the result obtained by 1.03. Anticoagulants citrate, fluoride and heparin, as a general rule, are not indicated because they interfere with methodologies commonly used in the laboratory *[6,10]*.

Total and HDL cholesterol assays can be done in non fasting conditions for the variation is negligible compared to the fasting ones.

Sample Storage

The serum should be separated from the clot at most within 2 to 3 hours of collection. The sample stored at 4°C is stable between 3 and 5 days for all routine determinations *[1,8,11]*. The highest reproducibility is obtained with the dosage on the day of sample collection. Longer storage processes require freezing of the sample at 20ºC or preferably -70ºC and some studies show loss of lipoprotein stability in these conditions.

Analytical procedures

The main difficulties in the quantification of lipids are linked to their insolubility in water, and to the fact that lipoprotein particles present a dynamic plasma metabolism, not

characterizing a single molecule, but a set of molecules with similar densities and compositions.

Ultracentrifuge

In this process lipoproteins are separated by density difference in an intense gravitational field. The separation process requires adjustment of the density (d=1,006 g/mL) of the medium and an ultracentrifuge capable of producing speeds of 10,000 X g for 16 to 24 continuous hours. Analysis of a single sample can take up to 3 working days. This technique is considered as a reference method for the quantification of various lipoproteins, and the current classification (by density) of lipoproteins (Kilomicrons, VLDL - Very Low Density Lipoprotein, IDL, LDL and HDL) derives from this procedure. This process is not suitable for the daily laboratory routine because it depends on sophisticated equipment, requires expensive technical resources and due to the length of the process *[6]*.

Electrophoresis

Proteins (and lipoproteins) become negatively charged when placed in a medium with a pH higher than its isoelectric point and migrate to the opposite pole of their charge (positive pole) when subjected to an electric field. This process allows separating lipoproteins by the charge of the protein fraction. Using cellulose acetate tapes or agarose gel as support, three fractions are separated into normal serum: beta (LDL), pre-beta (VLDL) and alpha (HDL), and the designation of the same lipoprotein by the ultracentrifugation process is in brackets. Kilomicrons when present are positioned at the point of application.

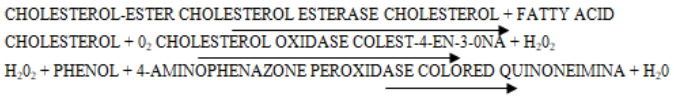
After electrophoretic separation, the fractions are evidenced with lipid dyes such as fat red 7B, followed by densitometry for the quantification of each fraction. The result should be interpreted as semiquantitative and evaluated in conjunction with the densitometric tracing (electrophoretic profile). This procedure, also known as lipidigram, is not a routine procedure. Its usefulness is limited to the detection of Fredrickson's type III dyslipidemia, by the identification and quantification of abnormal IDL (large-beta) and in the characterization of the absence of lipoproteins by genetic errors *[6]*.

A modification in this procedure allows the use of a reagent for cholesterol staining (enzymatic cholesterol) in fractions after electrophoretic separation, allowing the quantification of cholesterol present in HDL, LDL and VLDL fractions (i.e. HDL-c, LDL-c and VLDL-c-Very Low Density Lipoprotein Cholesterol). This procedure has a high cost, is slow and lacks precision, especially when HDL-c levels are lower than 45 mg/dL, and is not recommended for routine.

Determination of TC

The procedure that uses cholesterol esterase/cholesterol oxidase enzyrs coupled to a chromogenic reaction (Trinder reaction) is the method used by most laboratories *[3]*.

Reaction



This methodology and its variants present as main advantages the simplicity of execution; the ease of automation; high accuracy, sensitivity and linearity; low cost and non-toxic stable reagents. This procedure also allows the quantification of HDL-c. Among the most frequent interferers are very high levels of bilirubin and hemoglobin (hemolysis) by staining these compounds; and intense lipemia, due to the turbidity of the reaction medium.

The main problems in the accuracy of the results obtained with these procedures have two origins: the calibration system and the quality of the reagents available on the market *[12-14]*.

Cholesterol, as a lipid, is insoluble in water and the reaction system with enzymes is essentially aqueous. The use of primary patterns of esterified or free cholesterol implies the need for solubilization of these compounds in water by the addition of a solvent that can exert an inhibitory effect on the enzymatic reaction. Another fundamental problem is that a primary pattern (cholesterol only) does not reflect the conditions of the patient's sample, in which cholesterol is solubilized in lipoproteins and in the presence of many other compounds (effect of the serum matrix) that can interfere with its reactivity. The best way to calibrate the enzymatic methodology is the use of a human serum pool that was measured by a reference method (which characterizes this pool as a secondary standard). The North American CDC and NCEP have established as reference method the chemical procedure in which cholesterol is extracted in organic solvent, saponified to release fatty acids and the chromogenic reaction of Liebermann-Burchard under controlled conditions is applied. Serum samples quantified by this method in reference laboratories are offered to verify accuracy (true value) in national quality control programs. In our country we do not have this procedure. The best proposal for cholesterol calibration in national clinical laboratories may be the use of lyophilized commercial calibrators, based on human serum, obtained from suitable manufacturers. The main difficulty for the indication of the generalized use of these calibrators is in the fact that the lyophilization process alters lipoproteins and the reconstituted lyophilized serum becomes cloudy. To eliminate this undesirable effect manufacturers add clarifying products (sucrose, detergents, emulsifiers) that may cause changes according to the formulation of the reagent (kit) used for TC dosage.

It is important that in addition to calibration, the methodology is validated with the use of control sera with low, normal and high values. Controls with low values (40-50 mg/dL) for TC are important in monitoring the sensitivity of the reaction due to the use of the same reagent for the quantification of HDL-c. The controls of high values evaluate the linearity of the method and the performance of enzymes and reaction system for

samples with pathological levels.

Participation in external quality control programs is essential in obtaining quality results, particularly in lipid tests.

We do not have a policy on quality of reagents for the clinical laboratory at national level. The lack of regulation in this area allows reagents to be marketed without the degree of accuracy and accuracy required for testing. This fact emphasizes the importance of choosing laboratories that excel in the quality of their procedures.

Determination of HDL-c

The quantification of cholesterol in the HDL fraction (HDL-c) is performed in the routine of the clinical laboratory through the use of selective precipitants or more modernly by the use of the reaction for TC dosage with modifications in enzymes and in the reaction system (direct HDL-c).

HDL-c with selective precipitation

Precipitating agents (polyanions) heparin, dextran sulfate (molecular weight=50,000) and sodium phosphotungstat in the presence of a divalent cation-manganese or magnesium selectively precipitate lipoproteins containing apo B (Kilomicrons, VLDL, IDL, LDL and Lp(a)). Precipitate is separated by centrifugation and cholesterol is quantified directly in the supernatant (which contains the HDL fraction) through enzymatic methods *[6,8,12,14]*.

The most commonly used precipitating agents are:

a. Heparin-$MnCl_2$
b. Dextrana sulfate-$MgCl_2$
c. Phosphotunqstat-$MgCl_2$

Precipitation with manganese heparin-chloride was used in most studies that established the relationship between atherosclerotic disease risk and HDL levels. The use of this procedure declined when the interference of this precipitant on the enzymatic reaction was evidenced, which became routine to quantify cholesterol. Changes in the concentrations of this precipitant and in the formulation of enzyme reagents for cholesterol eliminated this interference and this modified reagent has a good correlation with the ultracentrifugation method.

The most commonly used selective precipitants in the routine are magnesium dextran-chloride sulfate and magnesium sodium-chloride phosphotungstat. This preference stems from the good compatibility of these agents with enzymatic methods, their high stability and because they form a firm sediment especially in the presence of lipemic samples. These precipitants are very little affected by the weather and temperature during the precipitation process, which makes them robust for use in the routine. Dextran sulfate and phosphotungstat have HDL-c values about 5% lower due to precipitation (co-precipitation) of small but significant amount of HDL when compared to ultracentrifugation.

Lipemia is one of the most frequent causes of loss of accuracy. In this situation there is an elevation in VLDL plasma and/or kilomicrons and therefore a much larger amount of apo B. The concentrations employed by precipitating agents are insufficient for the complete precipitation of all apo B present in the lipemic samples. In this case a common analytical error is the overestimation of HDL-c by the quantification of cholesterol present in lipoproteins other than HDL, which were not effectively precipitated. The influence of lipemia is complex, because the phenomenon described above is associated with a well-established correlation between the elevation of TG (Iipemia) and the reduction of HDL-c levels, which make the sample more subject to errors due to methodological sensitivity (quantification of low concentrations) and increase the VCa%. Procedures such as dilution of lipemic samples to promote complete precipitation of apo B can be used. However, in our experience lipemic sample dilutions greater than 1:2 performed in saline greatly reduce the reliability in the result and are not recommended. Therefore, lipemia when intense is a limiting factor for the use of selective precipitation in the quantification of HDL-c, in our opinion.

The observations in a previous topic, for the determination of TC, in relation to the calibration processes of the methodology are also valid for the quantification of HDL-c. Calibrators and controls based on human serum are preferred. The verification of linearity and methodological sensitivity in low HDL-c values is of paramount importance since the values for clinical decision are around 35 mg/dL, much lower therefore than the TC levels. The quality of the spectrophotometer (or automatic analyzer) and its maintenance status are fundamental to obtain accurate and reproducible results *[13]*.

Direct measurement of HDL-c

This method eliminates the separation phase of lipoproteins, and uses a single reagent. The assay is based on the enzymatic reaction for the determination of TC, in which the enzymes cholesterol esterase and cholesterol oxidase were modified by chemical treatment controlled with polyethylene glycol (PEG). These PEG-modified enzymes have a selective catalytic activity by the HDL particle in the serum and low by the other lipoproteins. Sulfated alpha-cyclodextrin was also added to the reaction medium, which reduces cholesterol reactivity from lipoproteins other than HDL, increasing the specificity of the method *[14,15]*.

This procedure has as advantages the use of only 4 microliters of sample in automated system and the elimination of the sample processing step (precipitation). Harris N, et al. *[16]* evaluated this methodology by comparing it to the reference procedure and observed an analytical coefficient of variation ≤ 4.1% and a total error within acceptable values by the NCEP (see Table 3). Another important point reported by these authors was the absence of interference in samples with triglyceride levels as high as 1,800 mg/dL.

Only one reagent with this methodological principle is available in Brazil to date and a small number of scientific papers on interfering and methodological problems are

available. The simplicity, speed of determination, the elimination of the precipitation stage (and its indirect costs) makes this a promising method to achieve the preference of clinical laboratories.

LDL-c determination

The quantification of cholesterol concentration in LDL lipoprotein (LDL-c) can be estimated by the Friedewald equation, either quantified by selective precipitation methods or even more recently with the use of lipoprotein separation with specific antibodies *[17]*.

LDL-c calculated by the Friedewald Equation: in this process LDL-c is estimated by the use of the empirical equation:
LDL-c=TC-(HDL-c+TG/5)

The triglyceride/5 ratio estimates the cholesterol present in the VLDL fraction (VLDL-c) when the values are quantified in mg/dL.

The use of the formula proposed by Friedewald is the most used process in clinical laboratories and presents in its favor: simplicity and the fact that the values obtained from epidemiological surveys that characterize the correlation and LDL-c levels and risk of coronary atherosclerotic disease were obtained with this equation *[18]*.

High triglyceride levels (≥ 400 mg/dL) limit the use of this equation. This limitation is characterized by the loss of correlation between the calculated values and those obtained by ultracentrifugation (reference method). Triglyceride values < 400 mg/dL have a good correlation with the reference method when the sample does not contain significant amounts of kilomicrons or a modified VLDL (β-VLDL, β-floating lipoprotein or wide beta) that characterizes type II hyperlipoproteinemia, of very low frequency in the general population. Therefore, fasting (avoiding the presence of kilomicrons) is essential for estimating LDL-c by calculation *[10]*.

Another disadvantage of estimating LDL-c by calculation that should be emphasized is that this measure depends on the quantification of TC, HDL-c and TG, thus embedding the biological and analytical variability of all components of the formula in the calculated LDL-c result. It is worth reviewing the wide intra individual biological variability of TG, discussed at the beginning of this review, to highlight the magnitude of this effect on this parameter.

The result obtained for LDL-c calculated by the Friedewald equation also includes amounts of other non-LDL lipoproteins present in the sample. These lipoproteins (basically IDL and Lp(a)) are atherogenic and present in very low concentration in the general population *[19]*. However, in some hyperlipemic samples, especially in patients with coronary artery disease, the contribution of these lipoproteins may be significant in the composition of the LDL-c result.

Recently new formulae have been presented in the literature and among all of them Martin/Hopkins formula has proven to be the more accurate for it takes into consideration each and every TG concentrations up to 800 mg/dL *[20-25]*.

Direct quantification of LDL-c

Direct determination of LDL-c can be performed by selective precipitation of LDL at its isoelectric point with polyvinylsulfate or sodium hepatin/citrate. The cholesterol of the supernatant containing VLDL and HDL is quantified by enzymatic methods and LDL-c is estimated by the calculation:

LDL-c=TC -cholesterol in the supernatant (VLDL+HDL).

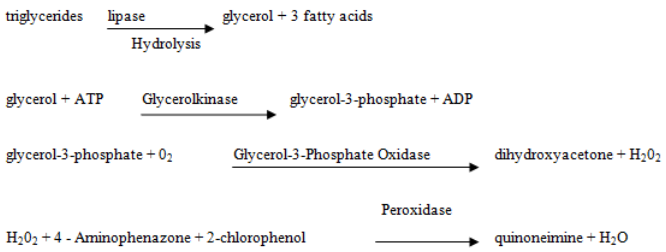
This procedure is interfered with triglyceride levels and so far there is no indication that it produces clinically better results than those obtained by calculation.

Another methodology promotes the separation of LDL through solid phase immune capture. In a matrix will be fixed specific antibodies for HDL and VLDL that bind to these lipoproteins and retain them in the matrix, and by filtration one obtains the sample that contains only LDL, which is quantified by enzymatic methods. This methodology is recent and there is little literature available on methodological interferences and problems. A disadvantage already characterized is the high cost of the procedure.

Determination of TG

The most used procedure in the clinical laboratory routine is the colorimetric enzymatic methodology that is based on the hydrolysis of TG by lipases and quantification of glycerol released through auxiliary reactions and Trinder chromogenic reaction *[6,810]*.

Basic reaction



The method is simple to perform, fast, easily automatizable and with very good accuracy and linearity.

All methodologies available on the market for the quantification of TG are based on a chromogenic or U.V. reaction on the glycerol released. If the calibration of these methods is not performed with serum-based standards (some manufacturers recommend calibrating only with glycerol), the action and efficacy of the lipase enzyme is not evaluated and this is an important source of error.

Another point to be evidenced is that these methodologies embed in the result of TG the free glycerol present in plasma. These values are usually negligible (about 10 mg/dL), but in

some situations such as uncontrolled diabetes, after strenuous exercise, intake of glycerol-containing medications and in the rare pathology hyperglycerolemia these values may be significantly elevated. The recommendation is to make a white to discount the glycerol present. The reagents available on the market do not allow this practice at the time.

Calibration and quality control are essential, as in any biochemical determination, in particular because TG participate in the composition of LDL-c calculation.

Determination of apolipoproteins

Apolipoprotein determinations are gaining importance as good risk discriminators for coronary artery disease *[18,26]*.

The quantification of apoproteins can be performed through several procedures such as *[2,8]*:

- RIA (radioimmunoassay)
- ELISA (enzyme immunoassay)
- Immunonefelometry
- Immunoturbidimetry
- Radial immunodiffusion
- FIA (fluorescent immunoassays)

In the national context, determinations by nephelometry and turbidimetry predominate in clinical laboratories, especially due to the ease of process automation and in the case of immunoturbimetry that can be performed using equipment common to other trials *[26]*.

The specificity of the antiserum used in any methodological procedure characterizes the sensitivity, specificity and reproducibility of the test. Several problems are encountered in the production of antisera and in the calibration system of the methods. The main one is the genetic polymorphism of apolipoproteins, which mask antigenic determinants, making it difficult to obtain reagents (antisera) with a constant and stable reaction profile *[8,11]*.

An antiserum produced by an isoform does not react with all isoforms of a specific apolipoprotein. Therefore, hidden antigenic sites of apoprotein while it is present in lipoprotein, is a potent source of analytical error. The specificity of antiserum produced against apoprotein is highly dependent on how it was used as an immunizing agent (intact, purified or delipidated). The tendency to associate when diluted leads to problems in the use of calibrators or markers in certain immunochemical reactions. Due to this instability, the recommendation for the use of secondary calibrators is given *[11]*.

The problems with the quantification and standardization of these trials led the world scientific community to form an International Standardization Commission. A serum pool was sent to 100 laboratories for apo A-I and apo B dosing. In the period 1986-1987, the VC between laboratories was 24% and 15% for apo B and A-I, respectively. The main source of error was the lack of a common calibrator between laboratories and the second the "matrix effect" (effect of the other components present in the serum other than the element under test) associated with the lyophilized material.

The main characteristics of the methodologies for quantification of apolipoproteins are found in Table 6 *[8,27]*.

In nephelometry and turbidimetry procedures, common in laboratory routine, lipemia is the main methodological interferent where the turbidity generated can cause positive interference *[28]*.

Sample

Serum or plasma (EDTA) with 12-hour fasting. Plasma is not recommended for techniques such as immunonefelometry and immunoturbidimetry since fibrinogen is a potential interfering in the immunochemical reaction.

Stability of Samples

There are several recommendations found in the literature regarding the stability of apolipoproteins. We suggest that each laboratory follow the instructions of the manufacturer of the used kit.

Recommendations are that normo and hypertriglyceridemic sera can be stored at 4°C for a period of up to 8 days for determinations of apo A-I and apo B by immunoturbidimetry[29].

Quality control

The reagent manufacturer must provide the material for quality control. It is recommended, however, that all laboratories analyze a stable and suitable human material, with at least two levels (high and low) for apolipoproteins. Both pools should be analyzed in each patient battery. Each lab needs to establish its own operational boundary based on repeated analysis of quality control material and follow standard criteria used for other analytes in their laboratories.

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