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Test Utilization and Outcomes

Significance of various parameters derived from biological variability of lipoprotein(a), homocysteine, cysteine, and total antioxidant status

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Analytical and biological components of variability and various derived indices have been determined for lipoprotein(a) [Lp(a)], homocysteine (Hcy), cysteine (Cys), and total antioxidant status (TAOS) in ostensibly healthy adult Caucasians and in stable outpatients with an increased serum Lp(a). In healthy Caucasians, average intraindividual biological CVs (CV_b) were 20.0% for Lp(a), 9.4% for Hcy, 5.9% for Cys, and 2.8% for TAOS, CV_bs being similar in men and women. In the outpatient group, CV_bs were comparable for Hcy, Cys, and TAOS, but significantly lower for Lp(a) (7.5% vs 20.0%; $P < 0.0001$). Moreover, a significant inverse relation between both biological and analytical CVs (CV_a) and serum Lp(a) concentrations was demonstrated. We conclude that average CV_a and CV_b values, and hence average derived indices, are adequate for Hcy, Cys, and TAOS, whereas individual values should be used for Lp(a).

In addition to the traditional lipid and lipoprotein risk factors for atherosclerotic disease, serum lipoprotein(a) [Lp(a)], plasma homocysteine (Hcy), and serum antioxidant concentrations are increasingly recognized as independent risk factors for atherosclerosis *[1–3]*.[5] Lp(a) is a lipoprotein particle that resembles LDL with a disulfide-linked apolipoprotein(a) [apo(a)] side chain. Apo(a) is structurally related to plasminogen, although it has no plasminogen activity, and, so far, its physiological function has not been unraveled. Lp(a) is mainly synthesized by the liver, its concentrations being mostly genetically determined and fairly constant throughout an individual's life *[1, 4, 5]*. Across populations, mean and median Lp(a) concentrations are lower in Caucasians than in people whose ancestors originated in Africa *[6]* or the Indian subcontinent *[7]*. However, high average concentrations of serum Lp(a) are observed in Caucasians with a family history of premature ischemic heart disease *[1]*. Although its evolutionary conservation suggests some selective advantage during some stage of man's evolution, it is hypothesized that in our present condition Lp(a) may increase the likelihood of thrombosis occurring on atheromatous plaques, because of its plasminogen resemblance.

Hcy is derived from the intracellular metabolism of methionine and is exported into plasma where it circulates primarily in oxidized form (i.e., Hcy and Cys–Hcy disulfide) and bound to proteins. Concentrations of total Hcy are increased in 15–40% of patients with coronary, cerebral, or peripheral arterial diseases *[2, 8]*. Mechanisms that may relate to the pathogenesis of atherothrombosis in hyperhomocyst(e)inemia are the change in hemostatic condition from antithrombotic to thrombogenic, the increased incorporation of Lp(a) into fibrin, and the increased oxidation of LDL *[2, 8]*.

The oxidation hypothesis of atherosclerotic disease emphasizes the causal role of oxidized lipoproteins in atherogenesis *[9]*. If decreased antioxidant concentrations accelerate lipoprotein oxidation and hence atherosclerotic disease, detection of a decreased total antioxidant status

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[5] Nonstandard abbreviations: Lp(a), lipoprotein(a); Hcy, homocysteine; apo(a), apolipoprotein(a); TAOS, total antioxidant status; CV_b, within-subject or intraindividual biological CV; CV_a, analytical CV; AG, analytical goal; CV_g, between-subject or interindividual biological CV; RCV, reference change value; and NS, number of specimens.
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(TAOS), as measured by antioxidant-mediated quenching of the absorbance of a radical cation *[10]*, may prove to be a valuable test.

To date, data on biological variation are absent for Hcy (and Cys) and TAOS, and abundant but conflicting for Lp(a) *[11–19]*. In the case of Lp(a), earlier estimates of the biological intraindividual CV (CV_b) showed a 7% week-to-week variation *[13]*, whereas in the ARIC study *[14]* the CV_b was estimated to be as low as 2.9%. More recent studies *[15–17]* reported average CV_bs of 7.6%, 10%, and 18%, whereas Marcovina et al. *[18, 19]* found the estimated CV_b to be highly variable (range 3–51%) and to have a systematic inverse relation with the Lp(a) concentration. Possibly, the highly skewed Lp(a) distribution and the 1000-fold interindividual spread in blood Lp(a) concentrations in Caucasians, in combination with the investigation of rather limited numbers of individuals, may have caused apparently conflicting data on intraindividual biological variation of Lp(a).

In the present study a comprehensive biological variability study for these analytes was carried out in a rather large group of healthy sex- and age-matched Caucasians. To ensure inclusion of an adequate number of individuals with high serum Lp(a), stable outpatients from the Lipid Clinic who repeatedly had Lp(a) mass concentrations >300 mg/L were enrolled. An experimental protocol that minimized preanalytical and analytical sources of variability was used. The aims were: (*a*) to estimate, in healthy and in chronically diseased but stable Caucasians, the biological variation of Lp(a), Hcy, Cys, and TAOS around the intraindividual homeostatic setpoints as well as the relation between the biological intraindividual variation and the analyte concentration; (*b*) to determine desirable analytical goals for these new or potential risk factors on the basis of intra- and interindividual biological variation *[11]*; (*c*) to gain a clear understanding of the value of conventional population-based reference values for these analytes *[12]*; (*d*) to gain a clear insight into significant and insignificant analyte changes in serial specimens *[12, 20]*; and (*e*) to calculate the minimum number of serial specimens needed to determine the "true" analyte concentration *[21]*.

Materials and Methods

STUDY SUBJECTS

Healthy volunteers. Fifty-four physically healthy Caucasians who had a stable life-style and diet, and who were not on lipid-lowering medication (27 men and 27 women; age range 21 to 46 years) were enrolled. Ongoing intake of supplementary vitamins, minor tranquilizers, or oral contraceptives throughout the study period was allowed, as well as occasional intake of an over-the-counter drug such as aspirin or paracetamol. All subjects were free of endocrine, metabolic, and immune disorders. None of the women with childbearing capability became pregnant during the study.

Outpatients with hyper-Lp(a) lipoproteinemia. Twelve Caucasian outpatients (5 men and 7 women; age range 22 to 69 years) from the Lipid Clinic of the University Hospital Rotterdam with an Lp(a) mass >300 mg/L were included. All patients were on a lipid-lowering diet for at least 3 months before enrollment.

STUDY PROTOCOL

Blood was collected biweekly at each of four visits per individual. Subjects were seen in standardized format at each occasion, i.e., they were asked to fast for 10 to 12 h before each visit, and to maintain their diet, life-style, and possible medication throughout the evaluation period. The design and intention of the study were thoroughly explained to all subjects and informed consent was obtained. All study subjects were interrogated on each of the four visits by one of two physicians who checked, by means of a predefined questionnaire, whether diet, life-style, smoking and drinking habits, and possible medication were maintained throughout the study. Height and weight were measured at the first visit, whereas body weight was checked at each subsequent visit. Fertile women were questioned about possible new pregnancies.

Venous blood was collected in the upright sitting position, immediately after individuals had been seated. Sampling was done between 0800 and 1000 by a single phlebotomist. Whole blood was collected for Lp(a) and TAOS *[22]*, whereas EDTA blood was collected for Hcy and Cys analyses *[23]*. Except for serum TAOS, which has limited stability according to the manufacturer, all four samples from one individual were analyzed in one run at the end of the study, to omit between-run analytical variation. The study protocol was approved by the Medical Ethical Committee of the University Hospital.

SPECIMEN HANDLING AND STORAGE

A strictly predefined protocol was used for specimen preparation: EDTA (1.5 g/L) blood tubes were put on crushed ice immediately after blood collection *[23]*. Whole-blood tubes were kept at room temperature until clotting took place. Both whole-blood and EDTA tubes were centrifuged at 4 °C (10 min, 1500*g*) within 1 h after blood drawing *[23]*. Serum and EDTA plasma were separated from the cells immediately after centrifugation. TAOS determinations were performed the same day, whereas the other aliquots were stored at −70 °C for combined analysis of all samples from one individual at the end of the study. Blood specimens for this study were gathered during a 3-month period.

LP(A), HCY, CYS, AND TAOS MEASUREMENTS

Lp(a) was measured in serum with an anti-apo(a) polyclonal capture ELISA from Biopool [TintElize lipoprotein(a), cat. no. 610220] *[4–6]*. Total Hcy and Cys were measured in EDTA plasma by using a rapid, isocratic HPLC method *[24, 25]*. Serum TAOS was applied on a Hitachi 911 analyzer (Boehringer Mannheim) with the

Randox kit and calibrator control material (cat. nos. NX2332 and NX2331, respectively). The TAOS assay measures the antioxidant-mediated quenching of the absorbance of a radical cation *[10]*.

TAOS analyses were done in duplicate at the day of sample collection, whereas Lp(a), Hcy, and Cys analyses from one individual were performed in duplicate within one run at the end of the study. The maximum sample storage time for frozen aliquots was 5 months. To further minimize analytical variation, a single technician performed all the assays and single lots of reagents were used. The between-run CV for the TAOS control material was 4.3% (n = 15 runs), corresponding to a between-run variance of 0.0025.

STATISTICS

If a quantity X exhibits biological variation such that the standard deviation in an individual is proportional to the homeostatic setpoint of the individual, then the quantity X is said to have a constant CV. The CV is a parameter expressing the proportionality of the standard deviation to the homeostatic setpoint. It can be defined similarly for the analytical variation around the true value of a specimen. When the CV of X is small, then it is well approximated by the standard deviation (σ) of ln$X$, with few assumptions required regarding the distribution of X or ln$X$. A proof of this is given in the *Appendix*.

The smaller the CV, the better the approximation will be. From a statistical point of view, it is better to directly estimate the CV by estimating the σ of ln$X$ as a single parameter (be it an approximation) than as a ratio of an estimated standard deviation to an estimated mean. For CVs <0.4 (i.e., <40%), the approximation by σ of ln$X$ is good enough for practical purposes, considering the efficiency gained by estimating it as a single parameter. In summary, analytical CV (CV_a) and CV_b can be estimated as σ_a and σ_b, respectively, after log transformation of the measured analyte values.

Hitherto, means, variances (σ^2), and CVs were estimated by using standard formulas. In case of TAOS, serial specimens were analyzed in separate runs, and thus σ^2_b was an estimator for the total of within-subject and between-run variance. To obtain a proper estimate of the within-subject variance for TAOS, the between-run variance derived from the TAOS control material was subtracted from σ^2_b.

Indices were derived from CV_a and CV_b data *[11, 12, 20, 21, 26]* as follows: analytical goal for imprecision (AG CV_a) $\leq$½CV_b; analytical goal for bias (AG bias) $\leq$¼$(CV_b^2 + CV_g^2)^{[1/2]}$ (CV_g is the between-subject or interindividual biological CV); index of individuality = $(CV_b^2 + CV_a^2)^{[1/2]}/CV_g$; reference change value (RCV) or critical difference = $2.77(CV_a^2 + CV_b^2)^{[1/2]}$; number of specimens required to ensure with 95% confidence that the mean result is within ±5% of the individual's homeostatic setpoint [NS (±5%)] = $1.96^2[(CV_a^2 + CV_b^2)/25]$; number of specimens required to ensure with 95% confidence that the mean result is within ±10% of the individual's homeostatic setpoint [NS (±10%)] = $1.96^2[(CV_a^2 + CV_b^2)/100]$.

DATA ANALYSIS

Data analysis was done separately for healthy subjects and outpatients after removing one sample from the TAOS data set because of in vitro hemolysis. All analyte results were transformed with natural logarithms. Variances calculated from the logarithmically transformed data were multiplied by 10 000 to convert the estimated standard deviations (σ) to the CVs expressed in percent.

Differences in biological variation between men and women in each group were tested by calculating ratios of the pooled variance of analytical and within-subject variance from one gender to the other gender. These calculated F-ratios were compared with the critical F-values (α = 0.05). Differences in biological variability between the healthy subject and patient groups were calculated in a similar way. Contribution of analytical variability to total test variability was calculated as: $\{[(CV_{a^2}/CV_b^2) + 1]^{[1/2]} - 1\} \times 100\%$ *[11]*. Concentration dependency of CV_b and CV_a vs the average analyte concentration was studied with linear regression analysis: $\ln CV = \ln\alpha - \beta \ln\text{mean} + \text{residual}$. The null hypothesis checked was that the slope β would be equal to zero. Confidence intervals (95%) for serum Lp(a) were calculated as ± 1.96 [(analyte concentration × 0.01 × $CV_a)^2$ + (analyte concentration × 0.01 × $CV_b)^2$]$^{[1/2]}$/number of specimens$^{[1/2]}$. Overall, a significance level of $P \leq 0.05$ was adopted.

Results

Table 1 summarizes the baseline characteristics of the study subjects as well as the analyte concentrations at the first visit. Mean intraindividual weight changes varied between −0.06 and +0.98 kg between subsequent visits (data not shown). Table 2 summarizes mean CV_a, CV_b, and CV_g, and percentile distributions of CV_b for Lp(a), Hcy, and Cys, whereas for TAOS only mean CV_b and CV_g are presented. In outpatients, similar average CV_b estimates were found, except for Lp(a) (7.5% vs 20.0%; F = 7.07; $df1$ = 162, $df2$ = 36; P <0.0001). CV_b did not differ between men and women for any of the analytes, either in healthy subjects (F <1.44 at α = 0.05; $df1$ = 81, $df2$ = 81) or in outpatients (F <2.18 at α = 0.05; $df1$ = 15, $df2$ = 21 or F <2.37 at α = 0.05; $df1$ = 21, $df2$ = 15) (data not shown). On the basis of average CV_a and CV_b values, we found that <10% of the total test variability was analytical for Lp(a), Hcy, and Cys, whereas for TAOS up to 98% of the observed test variability was analytical. Table 3 displays the average derived indices for each analyte studied. Analytical goals for imprecision, based on average CV_b, were achieved for all analytes except for TAOS *[11, 12, 26]*. All analytes had marked individuality (index <0.6) *[12]*, demonstrating that the use of population-based reference values is inadequate for their interpretation. In the healthy subject group average, applicable

Table 1. Baseline characteristics of the study subjects.

Mean ± SD (median) or percentage	Healthy participants (n = 54)	Outpatients with Lp(a) >300 mg/L (n = 12)
Male/female ratio (%)	50/50	42/58
Smoking behavior		
Nonsmokers (%)	61	58
Ex-smokers (%)	20	33
Smokers (%)	19	8
Alcohol use, no/yes (%)	19/81	0/100
Regular physical exercise, no/yes (%)	46/54	67/33
Lipid lowering medication, no/yes (%)	100/0	17/83
Age (years)	32.7 ± 6.6	47.0 ± 13.8
Height (cm)	174.9 ± 8.7	169.0 ± 9.3
Weight (kg)	74.4 ± 12.4	73.5 ± 14.4
Body mass index (kg/m^2)	24.4 ± 3.9	25.6 ± 3.8
lnLp(a) (mg/L)	4.491 ± 1.385 (4.411)	6.578 ± 0.590 (6.686)
Homocysteine (μmol/L)	11.55 ± 3.65 (10.78)	15.37 ± 8.35 (13.03)
Cysteine (μmol/L)	235 ± 36 (232)	260 ± 32 (257)
TAOS (mmol/L)	1.334 ± 0.082 (1.334)	1.245 ± 0.077 (1.231)

differences required for two results to be significantly different at $\alpha = 0.05$ were 60% for Lp(a), 28% for Hcy, 17% for Cys, and 9% for TAOS *[20]*. Further, Table 3 reveals that for proper assessment of coronary artery disease risk in the population by means of the assays used, multiple serial specimens are needed if the observed value should be within, e.g., 5% of the true value.

Concentration dependency of $\ln(CV_a)$ and $\ln(CV_b)$ was studied in the healthy subject group for all analytes (data not shown). None of the parameters showed concentration dependency, except ln[Lp(a)], the slopes being (borderline) significantly different from zero ($P = 0.04$ for CV_a vs subject mean per visit; $P = 0.07$ for CV_b vs overall subject mean). After taking the antilogarithm the equations were: $CV_b = \{[42.9/Lp(a)^{0.31}] \times \exp[(0.82)^2/2]\}$ and $CV_a = \{[12.2/Lp(a)^{0.35}] \times \exp[(0.93)^2/2]\}$. Notable is that the slopes were comparable, whereas the intercept with the *y*-axis was 3.5 times higher for CV_b compared with CV_a. Fig. 1 illustrates the impact of changing CV_a and CV_b values across the Lp(a) concentration range on the Lp(a) test variability, analyzing one, three, and five serial specimens. If at least three serial specimens are analyzed per individual, the observed Lp(a) result is within ±10% of the true value if the Lp(a) concentration is >500 mg/L. Below 500 mg/L the test uncertainty runs up quickly because of increasing biological and analytical CVs. If only one specimen is analyzed, the observed Lp(a) value is within ±15–20% of the true value, even at Lp(a) concentrations >500 mg/L. Below 500 mg/L the confidence limits increase even more dramatically. At 300

Table 2. Overall within-run analytical and intra- and interindividual variation of Lp(a), Hcy, Cys, and TAOS in healthy subjects and in stable outpatients with hyper-Lp(a) lipoproteinemia.

		Mean CV, %			CV_b, %							
					Percentiles					Range		Contribution of mean CV_a to total test variation, %
Parameter	Analyte conc. range at first visit	CV_a	CV_b	CV_g	10	25	50	75	90	Min.	Max.	
Healthy subjects (n = 54)												
Lp(a), mg/L	2–1105	8.6	20.0	ND	1.9	6.6	10.8	23.4	30.7	0.0	58.7	8.9
Hcy, μmol/L	7.6–30.4	3.7	9.4	23.9	2.4	3.8	7.9	11.4	14.4	0.0	26.1	7.3
Cys, μmol/L	140–334	1.7	5.9	12.3	1.9	3.3	4.7	7.1	9.2	0.0	11.1	4.1
TAOS, mmol/L	1.174–1.498	4.3	2.8	4.5	—	—	—	—	—	—	—	97.8
Outpatients (n = 12)												
Lp(a), mg/L	201–1715	3.1	7.5	ND	1.3	3.8	6.1	9.5	13.9	1.1	14.8	8.3
Hcy, μmol/L	8.4–37.1	4.0	9.3	ND	3.1	5.7	6.3	9.9	17.0	2.9	17.9	8.8
Cys, μmol/L	214–335	2.0	6.0	12.9	1.1	2.9	5.5	8.2	9.0	0.0	9.0	5.4
TAOS, mmol/L	1.149–1.416	4.3	1.0	3.5	—	—	—	—	—	—	—	—

ND, no data (σ >0.40).

CVs were calculated from duplicate measurements at four biweekly visits, after logarithmic transformation of the analyte concentrations, Lp(a), Hcy, and Cys analyses being performed in one run at the end of the collection period, and TAOS analyses, for reasons of limited stability, being performed at the day of sample collection, i.e., in four different runs. For TAOS the CV_a represents the between-day CV of the assay, calculated from the TAOS control material. For TAOS, only average CV_b and CV_g were estimated, by correcting for the average between-day variance of the TAOS control.

Table 3. Indices derived from biological variation data for Lp(a), Hcy, Cys, and TAOS in healthy subjects and in stable outpatients with hyper-Lp(a) lipoproteinemia.

Parameter	AG CV_a, %	AG bias, %	Index of individuality	RCV, %	NS (±5%)	NS (±10%)
Healthy subjects (n = 54)						
Lp(a), mg/L	(10.0)	ND	(0.15)	(60)	(73)	(18)
Hcy, μmol/L	4.7	6.4	0.42	28	16	4
Cys, μmol/L	2.9	3.4	0.50	17	6	1
TAOS, mmol/L	1.4	1.3	0.62	9	5	1
Outpatients (n = 12)						
Lp(a), mg/L	(3.8)	ND	NA	(23)	(10)	(3)
Hcy, μmol/L	4.6	ND	NA	28	16	4
Cys, μmol/L	3.0	3.6	NA	18	6	2
TAOS, mmol/L	0.5	0.9	NA	5	4	1

NS (±5%), NS (±10%), number of serial specimens required to reduce uncertainty to within ±5% and ±10% of the true value, respectively; ND, no data; NA, not applicable.

Average indices were calculated on the basis of average CV_a, CV_b, and CV_g values, using the formulas presented in the text (Statistics). Such an approach is valid for Hcy, Cys, and TAOS, but not for Lp(a) because of concentration dependency of CV_a and CV_b. Therefore, average indices derived for Lp(a) are placed in parentheses and should be interpreted with caution.

mg/L, an internationally recognized though arbitrarily defined cutpoint for Lp(a) *[1]*, the confidence intervals range between ±21%, ±12%, and ±9% depending on whether one, three, or five serial specimens, respectively, were analyzed.

Discussion

This study reports comprehensive data about biological variability and certain indices derived from it for Lp(a), Hcy, Cys, and TAOS. So far, data on biological variation are absent for Hcy, Cys, and TAOS, and conflicting for Lp(a) *[13–19]*. According to Fraser, estimates of within-subject biological variation should be independent of (*a*) the population examined, (*b*) the age and the number of the subjects studied, (*c*) the locale where the study was conducted, (*d*) the health of the subjects, (*e*) the time scale, and (*f*) the analytical variability of the methodology used *[11, 12]*. However, as a result of the wide variation of published average CV_b estimates for Lp(a), Fraser's points of departures do not seem to be universally valid across different Lp(a) populations *[18, 19]*. Therefore we decided to investigate biological variation both in a large randomized sample of healthy Caucasians and in a selected hyper-Lp(a) lipoproteinemic patient group, enabling the study of eventual heterogeneity of within-subject variation.

In healthy Caucasians average CV_bs were 20.0% for Lp(a), 9.4% for Hcy, 5.9% for Cys, and 2.8% for TAOS (Table 2), mean CV_bs being similar in men and women for all analytes studied. In the outpatient group, comparable CV_b estimates were found for Hcy, Cys, and TAOS but not for Lp(a) (7.5% in outpatients vs 20.0% in healthy controls). Moreover, in accordance with Marcovina et al. *[18, 19]*, a systematic inverse relation was demonstrated

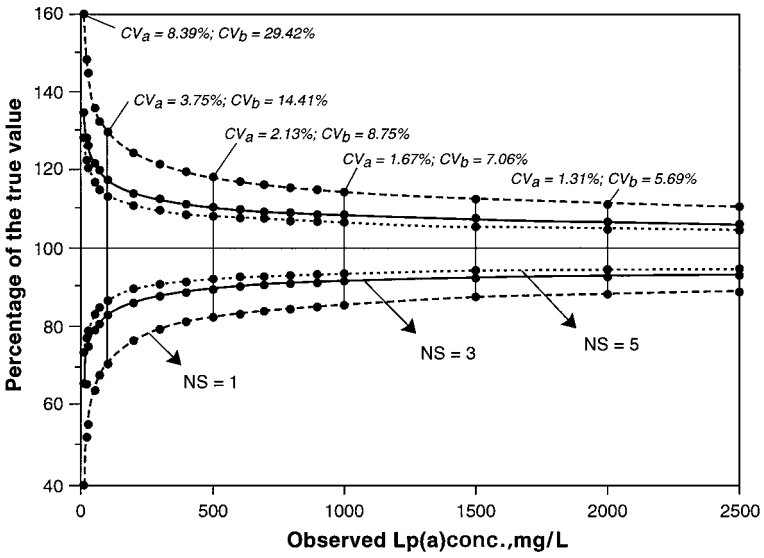

Fig. 1. Confidence intervals (95%) for the subject's true value of Lp(a) as percentage of the observed serum Lp(a) value for one, three, and five serial specimens.

between CV_a and CV_b and Lp(a) concentration. For the other analytes, no concentration dependency was found. Our data (*a*) illustrate the inadequacy of using average CV_b and CV_a values for Lp(a); (*b*) explain the controversy in the literature regarding intraindividual biological variability of Lp(a) *[13–17]* and corroborate the findings of Marcovina et al. *[18, 19]*; and (*c*) underscore the fact that the intraindividual biological variability of Lp(a) is greater than previously believed, especially in the low concentration range *[1]*.

Data on interindividual biological variation (CV_g) are presented if meaningful (Table 2). In general, interperson variability is determined by age, sex, diet, and genetics. In the case of Lp(a), interperson variability is mainly determined by genetics *[1]*, whereas diet and genetics may influence plasma Hcy concentrations *[2, 8, 23]*. Because the outpatient group represents a selected high-risk group, including individuals with both increased Lp(a) and Hcy concentrations (Table 1), CV_g data cannot be extrapolated from one study to another. Consequently, CV_g data are specific for the population studied and therefore are of limited value.

Several indices have been derived from the biological variability study. First, analytical goals for imprecision, having been the subject of a variety of approaches *[11, 12]*, were calculated. In this study the approach of Harris *[26]* was used, which states that maximum allowable analytical imprecision should be $\leq \frac{1}{2}CV_b$. Average goals for CV_a were met for Hcy and Cys, but not for TAOS (Table 3), average goals for CV_a being similar for Hcy and Cys in either study group. In contrast, for Lp(a) a single mean CV_a goal was not ubiquitously valid because of concentration dependency of the CV_b estimates. From the estimated regression lines that described the relation between CV_a and CV_b and Lp(a) concentrations, a fairly constant 3.5-fold difference between CV_a and CV_b could be demonstrated across the entire Lp(a) concentration range, signifying that the imprecision of the Lp(a) Biopool kit used is adequate at all Lp(a) concentrations. Therefore, we disagree with Pagani and Panteghini *[15, 21]*, who claimed that in practice the analytical goal for Lp(a) cannot be achieved with current assays. Notwithstanding the lack of international Lp(a) standardization and the fact that a different ELISA was used by these authors, the discrepancy with their data can be explained by the inappropriate use of average CV_b and CV_a estimates. Finally, although the CV_a goals were met for Lp(a) and Hcy (and Cys), one may object that the assays were performed under optimal conditions of variance as between-day variation was omitted. Yet between-day analytical CVs from routine practice in our laboratory of 7.1% at 67 mg/L, 4.2% at 213 mg/L, and 5.1% at 379 mg/L were achieved for Lp(a), whereas between-day CVs of 4.0% at 19.5 μmol/L and 3.2% at 52.2 μmol/L were obtained for Hcy, confirming the practical attainment of the analytical goals.

Second, desirable goals for average analytical bias were calculated (Table 3) *[11, 12]*. Documenting bias of routine assays necessitates the development of reference and (or) definitive methods and standardization programs for the analytes studied. So far, no international standardization has been reached.

Third, the utility of conventional population-based reference values was assessed by calculating an index of individuality in the healthy subject group (Table 3). The index gives a philosophical view on the interpretation of analyte data measured in healthy individuals and pathological changes in relation to reference intervals *[12]*. If the index is <0.6, then the use of reference intervals is of limited value in the detection of unusual individual results; if the index is >1.4, then reference values are of significant utility. In this study, all analytes had marked individuality, demonstrating that the use of population-based reference values is inadequate for their interpretation. This favors the adoption of cutpoints based upon relative risk of coronary artery disease.

Fourth, biological in addition to analytical variation data are also used for the critical evaluation of the significance of changes in results obtained from analysis of serial specimens *[12, 20]*. To interpret serial results objectively it is necessary to know the change that must occur before significance can be claimed. This RCV depends on both analytical and intraindividual biological variation, and holds only if all individuals have the same within-subject variation and if the analytical variation is constant across the concentration range. For Hcy, Cys, and TAOS, average RCVs for detecting significant changes in 50% of the individuals are presented in Table 3. In view of the concentration dependency of CV_a and CV_b for Lp(a), i.e., the observed variance reduction with increasing Lp(a) concentrations, mean CV_a and CV_b cannot be the basis for calculating the critical difference that is generally applicable in all individuals. So far, critical differences for Lp(a) were reported by one group *[15]*, the RCV being estimated as 29%, on the basis of a mean CV_a of 7.4% and a mean CV_b of 7.6%. According to our findings, critical differences for Lp(a) should be calculated on the basis of individual CV_a and CV_b values.

Finally, from the variation data obtained in this study one can estimate the number of specimens required to determine the individual's true homeostatic setpoint value *[21]*. Again, simple recommendations regarding the average number of specimens needed can be made for Hcy, Cys, and TAOS (Table 3), while such an approach is not valid for Lp(a) (Fig. 1). However, in light of the enormous interindividual concentration differences, we agree with Marcovina et al. *[18, 19]* that the CV_b of Lp(a) is not likely to be an important contributor to the misclassification of an individual's risk, unless the value is near the cutpoint of enhanced coronary artery disease risk.

Overall, the findings in the present study demonstrate that the understanding of the magnitude of the physiological variations that occur in Lp(a), Hcy, Cys, and TAOS

concentrations in serum or plasma is indispensable for proper use of these laboratory data for risk classification of patients with coronary artery disease. In essence, we demonstrated that average CV_a and CV_b estimates and mean derived indices are valid for Hcy, Cys, and TAOS, whereas individual values should be used for Lp(a). Second, the analytical performance of the Lp(a), Hcy, and Cys assays used is acceptable, taking into consideration the biological variation of these parameters, whereas the performance of the TAOS assay was insufficient.

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Appendix

A measured value X is supposed to have an expectation $E(X) = T$, the unknown true value of X, and a constant $CV(X)$. A simple model that implies these conditions is: $X = TU$, with U a random error term with mean $E(U) = 1$ and $SD(U) = \sigma$. This standard deviation is assumed to be small enough (e.g., $\sigma < 0.4$) that the probability of negative or zero values of U must be zero. No further assumptions about the distribution of U are made.

$CV(X) = SD(X)/E(X) = [(T\sigma)/T] = \sigma$, which is a constant single parameter. An approximate estimator for σ can be obtained by first taking the natural logarithm: $\ln X = \ln T + \ln U$, and then applying the so-called "delta method" *[27]* to the variance of $\ln U$:

$$var(lnU) \approx [d(\ln U)/dU]^2_{U=1} \times \mathrm{var}(U)$$

so that $\mathrm{var}(\ln U) \approx \sigma^2$, because $[d(\ln U)/dU]^2$ developed for $E(U) = 1$ equals unity *[27]*. Hence, $CV(X)$ can approximately be estimated by $SD(\ln X)$ within the same subject or within the same specimen under very general conditions.

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