Variability of Serial Lipoprotein-Associated Phospholipase A_2 Measurements in Post–Myocardial Infarction Patients: Results from the AIRGENE Study Center Augsburg

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BACKGROUND: Of the numerous emerging biomarkers for coronary heart disease (CHD), lipoprotein-associated phospholipase A_2 (Lp-PLA_2), an enzyme involved in lipid metabolism and inflammatory pathways, seems to be a promising candidate. Implementation of Lp-PLA_2 measurement into clinical practice, however, requires data on the reliability of such measurements.

METHODS: We measured Lp-PLA_2 concentrations by ELISA in blood samples drawn from 200 post–myocardial infarction patients (39–76 years) at 6 monthly intervals between May 2003 and February 2004, for a total of 1143 samples. We estimated analytical, within-individual, and between-individual variation, the critical difference, and the intraclass correlation coefficient of reliability (ICC) to assess the reliability of serial Lp-PLA_2 measurements.

RESULTS: The mean (SD) plasma Lp-PLA_2 concentration for the study participants was 188.7 (41.8) μg/L, with no significant difference between men and women. The analytical CV for Lp-PLA_2 was 4.4%, the within-individual biological CV was 15%, and the between-individual CV was 22%. The ICC was 0.66. An important part of the total variation in plasma Lp-PLA_2 concentration was explained by the between-individual variation (as a percentage of the total variance, 66.1%), whereas the within-individual variance was 31.3%. The analytical variance was as low as 2.6%.

CONCLUSIONS: Between-individual variation in Lp-PLA_2 concentration was substantially greater than within-individual variation. In general, our data demonstrate considerable stability and good reproducibility of serial Lp-PLA_2 measurements, results that compared favorably with those for the more commonly measured lipid markers.

A systemic inflammatory response represents an important feature in atherothrombogenesis *(1)*; therefore, measurement of plasma or serum concentrations of circulating inflammatory biomarkers could aid in identifying individuals at high risk for coronary heart disease (CHD).[4] A number of prospective studies of initially healthy individuals and patients with manifest atherosclerosis have convincingly demonstrated strong and independent associations between even slightly increased concentrations of various systemic markers of inflammation and a number of cardiovascular endpoints *(2)*. One of the various emerging biomarkers, lipoprotein-associated phospholipase A_2 (Lp-PLA_2), an enzyme that circulates primarily bound to LDL, seems to be a promising candidate, the measurement of which might improve our ability to identify patients at high risk. Indeed, a growing body of evidence from large prospective population-based and clinical studies suggests that Lp-PLA_2 is a promising and clinically relevant marker for CHD and probably also for stroke [for recent reviews, see *(3, 4)*]. Because of its ability to gen-

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[4] Nonstandard abbreviations: CHD, coronary heart disease; Lp-PLA_2, lipoprotein-associated phospholipase A_2; ICC, intraclass correlation coefficient of reliability; AIRGENE, Air Pollution and Inflammatory Response in Myocardial Infarction Survivors: Gene-Environment Interaction in a High Risk Group; MI, myocardial infarction; TC, total cholesterol; VC, variance component; VC_a, analytical VC; VC_b, between-subject VC; VC_w, within-subject VC; and IoI, index of individuality.

erate potent proatherogenic and proinflammatory compounds such as lysophosphatidylcholine and oxidized free fatty acids via hydrolysis of oxidized LDL, Lp-PLA$_2$ may be directly involved in the development of atherosclerotic plaques *(3)*. In contrast to other emerging biomarkers related to inflammation, Lp-PLA$_2$ is not an acute-phase reactant and thus is unaffected by systemic inflammatory processes (e.g., osteoarthritis and chronic obstructive pulmonary disease) *(5)*. Lp-PLA$_2$ may therefore represent a more specific marker of vascular wall inflammation. That Lp-PLA$_2$ is only minimally associated with systemic inflammatory and hemostatic markers *(6)* may reflect its independent impact on CHD. Moreover, considering that selective inhibitors of Lp-PLA$_2$ are under evaluation in clinical trials *(7)*, this enzyme may represent an attractive and novel therapeutic target for the treatment of atherosclerosis. Before the widespread use of Lp-PLA$_2$ testing in clinical practice can be recommended, however, several important requirements should be fulfilled (e.g., determination of population-based reference values) *(8)*. Recently, such cutoff points have been proposed, with values for men and women of between 230 μg/L and 250 μg/L and with desirable Lp-PLA$_2$ concentrations of $\leq$235 μg/L *(9)*. Furthermore, rigorous standardization of assays is necessary to ensure adequate reproducibility of Lp-PLA$_2$ measurements, and the imprecision of such assays should be low enough to enable a reliable and accurate assessment of future disease risk with only 1 or 2 samples. Data on the biological variation in Lp-PLA$_2$ concentration are also of considerable relevance, because they may be important for correctly classifying individuals over time.

Although variability data are available for several commonly measured biochemical analytes, such as lipid markers *(10)* and C-reactive protein *(11–13)*, data on the analytical and biological variation in Lp-PLA$_2$ measurements are still lacking. Therefore, we investigated analytical imprecision and intra- and interindividual biological variation in serial Lp-PLA$_2$ measurements made over a 6-month period for a cohort of post–myocardial infarction (post-MI) patients.

Materials and Methods

STUDY DESIGN AND POPULATION

The multicenter longitudinal AIRGENE (Air Pollution and Inflammatory Response in Myocardial Infarction Survivors: Gene-Environment Interaction in a High Risk Group) study has investigated the role of air pollution in eliciting inflammation in MI survivors in 6 European cities characterized by different climates, air quality, and lifestyles. The participating centers are Athens, Greece; Augsburg, Germany; Barcelona, Spain; Helsinki, Finland; Rome, Italy; and Stockholm, Sweden. The design and general protocol of the AIRGENE study have recently been described in detail *(14)*.

The present analysis is restricted to study participants from Augsburg, Germany, and includes 200 post-MI patients, ages 39–76 years, from whom approximately 6 blood samples were collected at monthly intervals between May 2003 and March 2004, for a total of 1143 samples. To be included in the study, all study participants had to fulfill the following inclusion criteria: (*a*) survival after an MI for 3 months to 6 years before study entry (corresponding to an MI occurring between 1997 and 2003) and (*b*) an age between 35 and 80 years. Post-MI patients were identified through the MONICA/KORA [Monitoring of Trends and Determinants in Cardiovascular Disease/Kooperative Gesundheitsforschung in der Region Augsburg (Cooperative Health Research in the Region of Augsburg)] coronary-event registry, and a major nonfatal MI was diagnosed according to European Society of Cardiology and American College of Cardiology criteria *(15, 16)*. The primary exclusion criteria were an MI and/or interventional procedure $<$3 months before the beginning of the study, an extended period of absence from the study area planned during the study period, or only 1 or no valid blood sample available for the patient. In addition, none of the study participants were allowed to have any of the following disorders associated with an acute-phase reaction: febrile acute infection, acute state of a chronic infection, or an inflammatory disease; underlying hematologic or malignant diseases; severe liver and renal disorders; surgery; or a major dental procedure during the 3 days preceding the clinical visit. The study was approved by the local authorities. Written informed consent was obtained from all patients at the first clinical visit after a detailed description of the study protocol was presented.

DATA COLLECTION

The health status of each patient was assessed at the first visit, and all study participants underwent a standardized interview carried out by a specially trained team of interviewers. Participants were asked about their medical history, including specific questions related to the history of CHD and other comorbidities. Data regarding current medications, sociodemographic characteristics, and lifestyle habits were also recorded. Finally, all study participants underwent a medical examination, including measurements of blood pressure and body mass index and a resting 12-lead electrocardiogram.

LABORATORY METHODS

Venous blood was drawn under standardized conditions at the clinical visits, which were usually scheduled both for the same time of day to minimize the effects of

circadian variation and on the same day of the week to minimize preanalytical variation. Samples were cooled down and stored at 4 °C until further processing, which was within 4 h of blood withdrawal. To obtain plasma samples, we centrifuged EDTA-treated blood at 2500*g* for 20 min and immediately aliquoted and froze the centrifuged samples at −80 °C until further analysis. No samples were inadvertently thawed during storage.

Lp-PLA_2 concentrations in the plasma were measured with a commercial Lp-PLA_2 ELISA (second-generation PLAC™ test; diaDexus) *(17)*. The interassay CV was 5.6% at an Lp-PLA_2 concentration of 239.1 μg/L and 6.27% at 469.0 μg/L. Total cholesterol (TC) was measured by routine enzymatic methods [cholesterol oxidase-peroxidase method (CHOD-PAP); Boehringer Mannheim]. HDL cholesterol was measured in the supernatant after apolipoprotein B–containing lipoproteins were precipitated with phosphotungstic acid and $MgCl_2$ (Boehringer Mannheim). All analyses were run in a blinded fashion.

STATISTICAL ANALYSIS

The distributions of baseline demographic features, clinical characteristics, and several biochemical markers in the study population are presented as the mean (SD) or as percentages. Residuals were checked for normality. Following concepts described by Fleiss *(18)*, Bland and Altman *(19)*, and Fraser and Harris *(20)* for the analysis of biological variation, we computed estimates for the 3 variance components (VCs) [VC_a (analytical), VC_b (between subjects), and VC_w (within subjects)], assuming nested normal random-effects models and using the SAS MIXED procedure (restricted maximum-likelihood method, RANDOM statement; SAS Institute). These components of variation (i.e., VC_a, VC_b, and VC_w) were then transformed into the corresponding CVs (CV_a, CV_b, and CV_w, respectively) by calculating the square root of the respective variance component estimate, dividing by the overall mean, and expressing the quotient as a percentage. We evaluated within-subject variation by calculating the critical difference (also called the reference change value or the 95% repeatability coefficient) according to Bland and Altman *(19)*, as follows: $1.96 \times (2)^{1/2} \times (VC_w + VC_a)^{1/2}$. We characterized the repeatability of measurements for comparing subjects or groups of subjects by calculating the intraclass correlation coefficient of reliability (ICC; also known as the reliability coefficient): $VC_b/(VC_b + VC_w + VC_a)$ *(21)*. In addition, we assessed the imprecision of measurements (analytical variation, or the error of the measurement process itself) by calculating the critical difference as $1.96 \times (2)^{1/2} \times VC_a^{1/2}$ and the ICC as $(VC_b + VC_w)/(VC_b + VC_w + VC_a)$. We also calculated an "index of individuality" (IoI), which was evaluated as the ratio: $(CV_w + CV_a)/CV_b$, with reference values ≤0.6 indicating little utility of the reference values for detecting unusual individual results and values ≥1.4 indicating the usefulness of reference values in many settings *(20, 22)*. The number of measurements required to achieve a reliability of 75% was computed with the Spearman-Brown prediction formula *(18)*. Finally, we also checked for outlying observation variances and subject variances with the Cochran test and checked outlying mean patient values with the Reed criterion *(20)*. The Cochran test was computed with the outliers package in R, version 2.4.0. All other statistical analyses were performed with the SAS software package, version 9.1 for Windows (SAS Institute).

Results

In this study, we included 200 MI survivors [mean age, 62.0 (9.0) years] who had provided at least 2 valid repeated blood samples. Table 1 summarizes the baseline demographic and clinical characteristics of the study population, as well as the mean values of several laboratory variables measured at the baseline visit. In this predominantly male population (82%), the mean time since the last MI was 2.1 (0.9) years, and 12.5% of the study participants had experienced recurrent MI. Furthermore, 51% of the MI survivors were defined as hypertensive, and about 90% of the study participants reported statin intake.

Blood samples were collected during a mean follow-up period of 7.3 months. The mean number of samples collected per individual was 5.7, and at least 6 blood samples were drawn from 179 study participants (89.5%). We drew a total of 1143 blood samples for Lp-PLA_2 measurement over approximately monthly intervals (mean, 47 days). We measured 13% of the samples in duplicate, however, so the overall number of Lp-PLA_2 measurements was 1291. The concentrations of Lp-PLA_2 in the plasma ranged from 55.1 μg/L to 417.0 μg/L, with a mean value (SD) over all measurements of 188.7 (41.8) μg/L. Women had slightly lower concentrations than men [(180.4 (33.6) μg/L vs 190.5 (43.2) μg/L]; however, this difference was not statistically significant ($P = 0.21$). After adjustment for age, sex, and statin intake, the mean Lp-PLA_2 plasma concentration for all study participants was 216.4 (40.1) μg/L [209.2 (31.6) μg/L for women and 220.5 (41.8) μg/L for men] (no statin intake, average age 61.9).

Table 2 demonstrates the calculated values for the measures of Lp-PLA_2 variation. Biological variation (VC_b and CV_b), intraindividual variation (VC_w and CV_w), analytical variation (VC_a and CV_a), the critical difference, the ICC and the number of measurements needed for a given ICC, and the IoI were estimated to assess the reliability of serial Lp-PLA_2 measurements.

Table 1. Demographic and laboratory characteristics of study participants.[a]

Characteristics	Post-MI Patients, n = 200
Age, years	61.9 (9.0)
Male sex, %	82.0
Myocardial infarction, %	
First MI	87.5
Repeat MI	12.5
Last MI to study, years	2.1 (0.9)
MI in family history, %	
Yes (mother and/or father)	30.5
No	58.0
Information incomplete	11.5
Family status married, %	81.0
School education, years	8.9 (1.9)
Body mass index, kg/m^2	28.8 (4.0)
Systolic BP[b], mmHg	128.4 (19.9)
Diastolic BP, mmHg	78.2 (10.6)
Physical activity, %	
Inactive	4.0
Partly active	13.0
Unregularly active	10.5
Regularly active	42.4
Trained	30.0
Smoking status, %	
Never smoker	31.0
Ex- or occasional smoker	69.0
Current smoker	0.0
Pack-years of cigarettes smoked	15.1 (21.5)
Alcohol intake, %	
None	14.7
Moderate	65.7
Heavy	19.7
History of diabetes, %	17.5
History of hypertension, %	51.0
Statin intake, %	89
Lp-PLA$_2$, μg/L	166.9 (47.4)[c]
Total cholesterol, mmol/L	4.68 (1.00)[c]
HDL cholesterol, mmol/L	1.24 (0.31)[c]

[a] Data are presented as the mean (SD) or a percentage.
[b] BP, blood pressure.
[c] Value at baseline (visit 1).

Table 2. Medium-term variability components for Lp-PLA$_2$.

Statistic	Lp-PLA$_2$
Subjects, n	200
Samples, n	1143
Measurements, n	1291
Overall mean (SD), μg/L[a]	188.7 (41.8)
Variance components	
VC_b	1744.9
VC_w	826.7
VC_a	69.1
CV, %[b]	
CV_b	22.1
CV_w	15.2
CV_a	4.4
Proportion of total variance, %	
Between subjects	66.1
Within subjects	31.3
Analytical	2.6
Within-subject variation	
Critical difference, μg/L	83.0
ICC	0.66
Analytical variation	
Critical difference, μg/L	23.0
ICC	0.97
Number of measurements needed[c]	2
IoI	0.89

[a] Overall mean was computed with a nested random-effects model to account for repeated measurements and duplicates.
[b] CVs calculated as the corresponding $(VC)^{1/2}$ divided by the overall mean for Lp-PLA$_2$.
[c] Number of samples required for a within-subject ICC of 0.75.

In this study, the CV_b was 22% (VC_b, 1744.9), the Lp-PLA$_2$ CV_w was 15% (VC_w, 826.7), and the CV_a was 4.4% (VC_a, 69.1). An important part of the total variance in plasma Lp-PLA$_2$ concentration was explained by between-subject variation, which was 66.1% of the total variance, whereas the within-subject variance was 31.3% of the total variance. Analytical variance constituted 2.6% of the total variance. In addition, the within-subject reproducibility of Lp-PLA$_2$ measurements (ICC, 0.66) indicates a good correlation of the serial measurements from the same individual during follow-up. The within-subject critical difference was 83.0 μg/L, indicating that, for post-MI patients, 2 Lp-PLA$_2$ measurements for the same patient at different times should be within 83 μg/L of each other 95% of the time. For the assessment of the measurement process, it is apparent that the ICC is large (0.97), i.e., very close to the maximum of 1. Furthermore, on the basis of our estimates, the value for the critical difference

for analytical variation demonstrates that 2 Lp-PLA_2 measurements for the same patient at the same time would be within 23.0 μg/L of each other 95% of the time. Thus, these results suggest that the measurement error in the Lp-PLA_2 assay is very low. We also calculated the necessary number of measurements for the present analysis and found that 2 serial Lp-PLA_2 measurements are required to achieve an ICC of 0.75. Finally, our calculation of IoI, which describes the relationship between within-subject variation and between-subject variation, yielded a value of 0.89.

In this study, we also applied 3 levels of outlier tests [described in *(20)*] and found that there were no outlying subject means according to the Reed criterion. The results of the Cochran test indicated 2 outlying observation variances and 2 outlying subject variances for Lp-PLA_2 concentration. Therefore, to investigate the sensitivity of our results, we repeated all analyses without these observations/subjects. (The results of such analyses are available as a Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue1.) In general, the exclusion of these values essentially did not change our primary results.

Discussion

We investigated medium-term variation (over a 6-month period) in serial measurements of Lp-PLA_2 concentration in 200 post-MI patients who participated in the AIRGENE study in Augsburg, Germany, and from whom 6 blood samples were taken at monthly intervals. In general, our data demonstrate considerable stability in and good reproducibility of the serial Lp-PLA_2 measurements.

The measurement error of the assay was extremely small (a low CV_a of 4.4% and a high ICC of 0.97). Indeed, only 2.6% of the total variation in Lp-PLA_2 concentration was ascribable to test imprecision. The CV_a for the present analysis compares well with the CV_as for commonly measured lipid variables (1.0%–1.9% for TC, 2.3% for apolipoprotein A-I, and 3.4% for triglycerides) *(10)*. Furthermore, a desirable goal for analytical imprecision *(23)*, which is usually set at less than half the average within-subject variation (CV_a <0.50 CV_w—i.e., $<7.6\%$ in our case), was also met in this study; however, optimum performance, which is defined as a CV_a <0.25 CV_w (i.e., $<3.8\%$ in our case) was not achieved here, although this preferable CV_a value was very close to the actual CV_a value of 4.4% calculated in the present analysis. Nonetheless, our data in general suggest that there is little measurement error in the analytical process.

Despite the small analytical variation in the method for Lp-PLA_2 assessment, our data further indicated that intraindividual variation is not negligible (CV_w, 15%). In agreement with our findings are the preliminary data from a small study on short-term Lp-PLA_2 variation that included data for 43 nonfasting healthy adults who had serum or plasma drawn at least 7 times over a 4-week period, for a total of 364 pairs of blood samples *(5)*. In this study, Wolfert et al. demonstrated 14.9% imprecision in repeated Lp-PLA_2 measurements from the same individual, and this estimate was nearly identical with the 15% CV_w estimate in our study. A comparison of the intraindividual variation in Lp-PLA_2 concentration with the intraindividual variation reported in various studies of commonly measured lipid variables showed that the CV_w for Lp-PLA_2 was slightly higher than for LDL cholesterol (6.5%, 7.4%, and 8.3% across different studies) *(5, 24)* and TC (5.8%, 6.0%, 8.8%, 9.3%) *(10, 11, 13, 24)* but was similar to or even better than for triglycerides (CV_ws of 15%, 21%, and 23%) *(5, 10, 24)*.

In addition, we measured the size of a change that would indicate a statistically significant difference between 2 measurements from the same patient at different times and found the critical difference between 2 measurements to be 83 μg/L. The Lp-PLA_2 ICC value of 0.66 further demonstrates the acceptable within-individual reproducibility of serial Lp-PLA_2 measurements. To achieve a reliability of 0.75, however, we recommend 2 serial measurements of Lp-PLA_2. A similar number of measurements was found to be needed for TC *(12)*, and the National Cholesterol Education Program guidelines thus recommend collecting 2 samples a week apart before deciding on a therapeutic strategy *(25)*.

The estimated CV_b for Lp-PLA_2 concentration was 22%, significantly greater than the CV_w value (15%); however, the CV_b value in general compared favorably with values reported for the more commonly measured lipid variables. For example, several studies have reported CV_b estimates of 11% *(10)*, 15% *(24)*, and 19% *(11)* for TC, and 28% for triglycerides *(10)*. In the study of short-term Lp-PLA_2 variation by Wolfert et al. *(5)*, between-individual variation for Lp-PLA_2 was 33%, compared with the 24% and 50% values for LDL cholesterol and triglycerides, respectively. Therefore, the CV_b value for Lp-PLA_2 in our study was even lower than that reported by Wolfert et al., despite the fact that different populations (post MI-patients vs healthy individuals) have been investigated and the possibility that biological variation might be higher in pathologic states than in the healthy state.

We also were interested in the utility of conventional reference values for Lp-PLA_2 in clinical practice (e.g., for monitoring, diagnosis, and screening) and calculated the IoI as a measure of how individuals vary with respect to the population distribution. Conven-

tional population-based reference values are of utility only when within-individual variation exceeds between-variation variation. In our study, however, the IoI for Lp-PLA$_2$ was 0.89, which is quite low, thereby demonstrating the marked individuality and limited utility of conventional reference values. Furthermore, a low IoI has several clinical implications. It may indicate that despite the possibility of detecting a highly unusual analyte concentration for a given individual, such values nonetheless would lie within the conventional population-based reference interval, thereby limiting the interpretation of even small changes from the set-point of this person. In such cases, it may be more important to accumulate data to evaluate the individual's own homeostatic set-point, which we recommend to be used instead of a population-based reference interval *(20)*. We note, however, that for most of the emerging cardiovascular biomarkers, within-individual biological variation is less than between-individual variation, e.g., IoIs not greater than 0.83 *(10, 12, 13)*.

Our study has several limitations that merit consideration. First, only post-MI patients were included; therefore, extrapolation to healthy individuals may not be justified, because the biological variation in cases of pathologic conditions may be significantly higher than in apparently healthy individuals. The only study to date that has assessed variation in Lp-PLA$_2$ measurements in healthy individuals *(5)* reported similar CVs. Moreover, only a small number of female participants (approximately 20%) were recruited in the present study, and the participants were mainly middle-aged individuals. Thus, sex- and age-specific comparisons of Lp-PLA$_2$ variation represent an issue that could not have been addressed in this study. In addition, we assessed only variation in serial Lp-PLA$_2$ measurements over a medium-term period (6 months). From an epidemiologic point of view, however, long-term variation may be even more important than short- or medium-term variation. Therefore, further studies with larger sample sizes are necessary to assess the long-term variation in Lp-PLA$_2$ measurements. Finally, we note that 90% of our study participants were receiving statin therapy, which lowered Lp-PLA$_2$ concentrations. This factor might have produced lower Lp-PLA$_2$ values and less variation in this biomarker in our population than in a population not exposed to statin treatment.

Our study also has several strengths of note. We collected all samples under extremely standardized conditions at each clinical visit to avoid preanalytical variation in Lp-PLA$_2$ concentrations. All samples were collected in the fasting state and mostly at the same time (98%) of the same weekday (95%) to minimize the impact of circadian and day-to-day variation.

In conclusion, the between-individual variation in Lp-PLA$_2$ concentration in blood samples taken repeatedly from post-MI patients over a 6-month period was substantially larger than within-individual variation, and the ICC value of 0.66 indicated the good reproducibility of serial measurements. In general, the values for measures of biological Lp-PLA$_2$ variation compared favorably with those of several "traditional" lipid variables; however, data on both seasonal and circadian variation in Lp-PLA$_2$ concentration and long-term variation are still lacking, and this paucity of data thus represents opportunities for further research.

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