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Biological variation of established and novel biomarkers for atherosclerosis: Results from a prospective, parallel-group cohort study

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ABSTRACT

Background: Biomarkers are a promising tool for the management of patients with atherosclerosis, but their variation is largely unknown. We assessed within-subject and between-subject biological variation of biomarkers in peripheral artery disease (PAD) patients and healthy controls, and defined which biomarkers have a favorable variation profile for future studies.
Methods: Prospective, parallel-group cohort study, including 62 patients with stable PAD (79% men, 65 ± 7 years) and 18 healthy control subjects (44% men, 57 ± 7 years). Blood samples were taken at baseline, and after 3-, 6-, and 12-months. We calculated within-subject (CV_I) and between-subject (CV_G) coefficients of variation and intra-class correlation coefficient (ICC).
Results: Mean levels of D-dimer, hs-CRP, IL-6, IL-8, MMP-9, MMP-3, S100A8/A9, PAI-1, sICAM-1, and sP-selectin levels were higher in PAD patients than in healthy controls ($P \leq .05$ for all). CV_I and CV_G of the different biomarkers varied considerably in both groups. An ICC ≥ 0.5 (indicating moderate-to-good reliability) was found for hs-CRP, D-Dimer, E-selectin, IL-10, MCP-1, MMP-3, oxLDL, sICAM-1 and sP-selectin in both groups, for sVCAM in healthy controls and for MMP-9, PAI-1 and sCD40L in PAD patients.
Conclusions: Single biomarker measurements are of limited utility due to large within-subject variation, both in PAD patients and healthy subjects. D-dimer, hs-CRP, MMP-9, MMP-3, PAI-1, sP-selectin and sICAM-1 are biomarkers with both higher mean levels in PAD patients and a favorable variation profile making them most suitable for future studies.

1. Introduction

Acute complications of atherosclerosis still represent a main cause of morbidity and mortality worldwide [1]. Cardiovascular risk assessment has substantially improved the management of patients with established atherosclerosis and of patients at risk for atherosclerosis. However, early detection, assessment of progression, and prediction of future complications are still challenging [2]. Current evidence underlines the pivotal role of inflammation in the pathophysiology, from initial endothelial dysfunction and development of fatty streaks, to the final step of plaque rupture and ensuing thrombosis [3]. Circulating markers of inflammation, endothelial dysfunction or thrombosis have been intensively investigated as possible diagnostic or prognostic biomarker for atherosclerosis [4–9].

Several established inflammatory and hemostatic biomarkers are increased in patients with lower extremity peripheral artery disease (PAD), a common clinical manifestation of atherosclerosis [10–14]. In a cohort of patients with coronary artery disease, those with concomitant PAD had higher inflammatory biomarkers than those without PAD [15]. The risk of cardiovascular events in PAD patients is 2 to 4 times higher compared to those without PAD, even after adjustment for the Framingham risk score [16]. Therefore, it has been suggested that PAD patients are an optimal study population to assess associations between circulating biomarkers and cardiovascular events [17].

Abbreviations: ABI, ankle–brachial index; ADMA, asymmetric dimethylarginine; CI, confidence interval; CV, coefficient of variation; EDTA, ethylene diamine tetra-acetic acid; eGFR, estimated glomerular filtration rate; $HbA1_c$, glycated hemoglobin; HDL, high-density lipoprotein; hs-CRP, high-sensitive C-reactive protein; ELISA, enzyme-linked immunosorbent assay; ICC, intra-class correlation coefficient; IL, interleukin; LDL, low density lipoprotein; MCP-1, monocyte chemoattractant protein-1; MDRD, Modification of Diet in Renal Disease; MMP, matrix metalloproteinase; oxLDL, oxidized LDL; PAD, peripheral artery disease; PAI-1, plasminogen activator inhibitor; sCD40L, soluble CD40 ligand; sICAM-1, soluble intercellular adhesion molecule; sP-selectin, soluble P-selectin; sVCAM, soluble vascular cell adhesion molecule; S100A8/A9, Ca^{2+}-binding protein S100A8/A9; TNF-α, tumor necrosis factor alpha.

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Before a potential biomarker can gain clinical utility, several steps have to be passed [18,19]. First of all, biomarker measurements should be reproducible and easy to perform [19,20]. Early phase biomarker studies should prove that there is a statistical association between the biomarker and the clinical state of interest, and that independent diagnostic or prognostic information is provided [18,20]. Advanced phase biomarker studies should provide evidence that measuring the biomarker has a positive health impact for the patient [18]. Randomized controlled trials or decision-analysis modeling are the best options for generating this evidence. Before decision-analysis modeling can be performed, exact biomarker variation in a real world setting needs to be known [18].

Reproducibility of serial measurements depends on analytical variation, day-to-day variation within subjects and variation between subjects [21,22]. The knowledge of these variations is important to estimate the informative value of a single time point measurement, to define a significant change over time or to define population based reference values [23]. Most studies evaluating the diagnostic or prognostic value of biomarkers report single time point measurements even though their biological variation is largely unknown [6–8,24].

Aim of the present study was to assess within-subject and between-subject biological variation of established and emerging biomarkers for inflammation, endothelial dysfunction or atherothrombosis over a one year period in patients with established, stable PAD of atherosclerotic origin and healthy controls, to compare the mean biomarker levels and variation between the two groups, and to define which biomarkers have a favorable variation profile for future studies.

2. Materials and methods

2.1. Subjects

We consecutively recruited two groups of subjects between 50 and 75 years of age in the outpatient clinic of a tertiary teaching hospital in Switzerland. The first group (PAD group) comprised patients with stable PAD from atherosclerotic origin. PAD was defined as an ankle brachial index (ABI) of less than 0.9, or previous revascularization for symptomatic PAD. The second group (control group) comprised healthy subjects with normal ABI values between 0.9 and 1.3 and no history of cardiovascular disease. Exclusion criteria were missing informed consent, severe renal failure defined as estimated glomerular filtration rate (GFR) ≤ 30 ml/min according to the MDRD formula [25], severe liver dysfunction (defined as chronic hepatic disease, e.g., cirrhosis, or previously documented biochemical evidence of significant hepatic derangement) [26], and atherothrombotic events — namely acute coronary syndromes, stroke, transient ischemic attack or any cardiovascular intervention within 4 weeks prior to study enrollment. Additional exclusion criteria for the control group were excessive smoking history (>10 pack-years) [27], diabetes mellitus (fasting plasma glucose ≥ 7 mmol/l, or hemoglobin $A1_c$ ≥ 6.5%), hypertension (ambulatory blood pressure > 140/90 mm Hg, or antihypertensive medication), hyperlipidemia (LDL-cholesterol ≥ 4.1 mmol/l) or history of venous thromboembolism.

2.2. Study design

The study was designed as a single center, prospective, parallel group cohort study. The study was approved by the local ethics committee and conformed with the principles outlined in the Declaration of Helsinki. Before participating to the study, all participants granted written informed consent.

All investigations took place in the morning on four occasions (visits), a baseline visit and three follow-up visits at 3, 6 and 12 months. At baseline, a vascular physician recorded demographic and medical characteristics using a standardized data collection form. At each study visit, a vascular technician measured the ABI and great toe–brachial index, and drew a pre-specified set of blood samples of 25 ml each for biomarker analysis.

2.3. Laboratory tests

Venous blood was drawn under standardized conditions in the morning after overnight fasting. Samples were collected in pyrogen-free tubes with and without ethylene-diamine tetra-acetic acid (EDTA). Considering the volume requirements for biomarker measurements, 4.9 ml of blood was taken for the preparation of serum, and 9.8 ml for EDTA, 2.6 ml for heparin, and 2.9 ml for citrate plasma. In order to exclude the possibility of platelet activation at the site of venous puncture, the first 2 ml of each blood draw was discarded. Serum was separated from coagulated blood by centrifugation at 1500 g for 10 min without cooling. Separated serum samples were immediately stored in a freezer at −80 °C. Analysis of biochemical makers were performed at Roche Center for Medical Genomics at F. Hoffmann-La Roche in Basel, Switzerland, with a single analytical run for each analysis. The Roche Central Sample Office provided blood collection kits with barcode labeled tubes to the investigators. Blood samples were shipped to the Roche Research laboratories by courier on dry ice. The samples collected from consented participants will be stored at the Biomarker Sample Repository (BSR) for 15 years after the end of the study (database closure). Thereafter, blood samples will be destroyed.

Research quality commercial kits were used to determine the levels of the selected biomarker candidates at Roche Research Laboratories. Fasting glucose, high-sensitive C-reactive protein (hs-CRP), total cholesterol, high-density lipoprotein (HDL), triglycerides, glycated hemoglobin ($HbA1_c$), D-dimer and serum creatinine were routinely analyzed at the study site. Low-density lipoprotein (LDL) was calculated according to the Friedewald-formula.

Asymmetric dimethylarginine (ADMA) was measured using the enzyme-linked immunosorbent assay (ELISA) from DLD Diagnostika, Germany. The level of E-selectin, matrix metalloproteinase-3 (MMP-3), and interleukins (IL-6, IL-8, IL-10, IL-21), Ca^{2+}-binding protein S100A8/A9 (S100A8/A9) and tumor necrosis factor (TNF-α) were measured using ELISA (IMPACT, Roche Diagnostics, Germany). Monocyte chemoattractant protein (MCP-1), plasminogen activator inhibitor (PAI-1) and soluble CD40 ligand (sCD40L) were measured using ELISA kits from R&D Systems, USA. MMP-9 levels was measured using fluorokine MAP Human MMP Base Kit from R&D Systems, USA with the corresponding Bead Kit and oxidized low-density lipoprotein (oxLDL) was measured using human oxLDL ELISA from Mercodia, Sweden. The concentrations of soluble intercellular adhesion molecule (sICAM-1), soluble P-selectin (sP-selectin) and soluble vascular cell adhesion molecule (sVCAM) were measured using human Adhesion Molecule Base kit from R&D Systems, USA with the corresponding Bead Kits.

2.4. Statistical analysis

Baseline characteristics and medication were compared between controls and PAD patients using unpaired *t*-tests and chi-square tests as appropriate. We used Q–Q plots to study the distribution of the biomarkers. Only sCD40L showed a normal distribution and was used untransformed in further steps. All other biomarkers showed a log-normal distribution and were used log-transformed in the outlier assessment and data analysis. We examined the data for outliers at the subject and group level, separately in the control and PAD group. Outliers within subjects were identified and removed using Cochran's C test, which examines the ratio of the maximum variance to the sum of the variances and compares this to the appropriate critical value in statistical tables [28]. Subjects with outlying mean values were identified and respective data points removed using Reed's criterion. This statistical test assesses the difference between the highest value and the next highest value (or the lowest value and the next lowest

value) and rejects the highest value (or lowest value) if the difference is larger than one-third of the difference between the highest and lowest values [29].

We calculated mean biomarker values with 95%-confidence intervals (95% CI) from restricted maximum likelihood random-intercept linear regression models that account for the correlation of measurements within subjects. For log-transformed biomarker values, the means and 95%-CIs were back-transformed to the original scale yielding geometric means with corresponding asymmetric 95%-CIs. *P*-values for differences between groups were calculated from Wald tests of mixed-effects linear regression models adjusted for age and gender using restricted maximum likelihood.

Within-subject and between-subject variances (Var_I and Var_G) of the two groups were derived from random-intercept linear regression models using restricted maximum likelihood. For normally distributed biomarker data, within-subject and between-subject coefficients of variation (CV_I and CV_G) were calculated by dividing the square root of the respective variance by the mean. For log-normal data, CV_I and CV_G were calculated according to the following formula: $CV = \sqrt{(\exp(Var) - 1)}$. The intra-class correlation coefficient (ICC) was calculated as the between-subject variance divided by the total variance. An ICC ≥ 0.7 indicates good reliability, between 0.5 and 0.7 moderate reliability and <0.5 poor reliability. The index of individuality equals within-subject CV divided by between-subject CV. The number of measurements needed to achieve a reliability (ICC) of 0.75 was calculated using the Spearman–Brown prediction formula: $0.75 * (1 - ICC) / (ICC * (1 - 0.75))$. The reference change value (RCV) between two consecutive biomarker measurements to be considered statistically significant at the 5% level is calculated according to the following equation for normal data: $RCV = 1.96 * \sqrt{2} * CV_I$. For log-normal data, the formula is $RCV_{up} = \exp(1.96 * \sqrt{2} * \sqrt{Var_I}) - 1$ for upward changes and $RCV_{down} = \exp(-1.96 * \sqrt{2} * \sqrt{Var_I}) - 1$ for downward changes [30].

For all measures of variability, a bias-corrected bootstrap 95%-CI was calculated. Bootstrap sampling was performed 5000 times, drawing samples at the subject level with sample size equal to the number of subjects.

All statistical analyses were performed using STATA version 13 (StataCorp., College Station, Texas, USA). *P*-values ≤ 0.05 and non-overlapping 95%-CI between the two groups were considered statistically significant.

Table 1
Baseline characteristics of study population.

	Control-group (n = 18)	PAD-group (n = 62)	*P*-value
Demographic data			
Age, years	56.6 ± 5.3	64.7 ± 6.5	<.001
Male sex	8 (44%)	49 (79%)	.004
Body mass index, kg/m^2	24.3 ± 3.5	28.2 ± 5.0	.003
Systolic blood pressure, mm Hg	118.6 ± 17.0	133.3 ± 20.0	.006
Diastolic blood pressure, mm Hg	73.2 ± 9.9	74.1 ± 8.9	.72
Heart rate, beats per minute	62.1 ± 7.8	71.0 ± 12.2	.004
Ankle–brachial index	1.19 ± .07	.81 ± .19	<.001
Toe–brachial index	.94 ± .18	.54 ± .23	<.001
Baseline lipid, diabetic and renal values			
Fasting glucose, mmol/L	5.2 ± .7	6.7 ± 1.9	.002
HbA1c, %	5.5 ± .5	6.7 ± 1.2	<.001
Total cholesterol, mmol/L	5.1 ± .7	4.7 ± 1.0	.12
Triglycerides, mmol/L	1.2 ± .5	2.1 ± 2.3	.09
HDL-cholesterol, mmol/L	1.7 ± .4	1.4 ± .4	.02
LDL-cholesterol, mmol/L	2.9 ± .7	2.3 ± .8	.009
eGFR, ml/min/1.73 m^2	86.6 ± 15.0	89.9 ± 28.3	.64
Risk factors and past medical history			
Hypertension	0 (0%)	49 (79%)	<.001
Diabetes	0 (0%)	30 (48%)	<.001
Smoking status			
Never smoker	14 (78%)	4 (6%)	<.001
Past smoker	2 (11%)	30 (48%)	.004
Current smoker	2 (11%)	28 (45%)	.009
Coronary heart disease	0 (0%)	21 (34%)	.004
Stroke or transient ischemic attack	0 (0%)	3 (5%)	.46
Concomitant medications			
Aspirin or clopidogrel	0 (0%)	56 (90%)	<.001
Aspirin and clopidogrel	0 (0%)	5 (8%)	.21
Vitamin K antagonist	0 (0%)	7 (11%)	.14
Statins	1 (6%)	45 (73%)	<.001
Other lipid lowering drugs	0 (0%)	8 (13%)	.11
ACE-inhibitors or ARB	0 (0%)	40 (65%)	<.001
Beta-blockers	0 (0%)	20 (32%)	.005
Calcium channel blockers	0 (0%)	15 (24%)	.02
Diuretics	0 (0%)	25 (40%)	.001

Note: Data presented as means ± standard deviation or number and (%). The *P*-value for a difference between the groups is calculated form an unpaired *t*-test for continuous variables and a chi-square test for categorical variables. ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker; eGFR, estimated glomerular filtration rate according to the MDRD-formula [25].

3. Results

A total of 62 patients with stable PAD of atherosclerotic origin and 18 control subjects were recruited (Table 1).

3.1. Biomarker levels

IL-21 and TNF-α have been excluded from the analysis as 81% and 65% of values were below the measurable threshold, respectively. After adjustment for gender and age, subjects with stable PAD had higher levels of hs-CRP, D-dimer, IL-6, IL-8, MMP-9, MMP-3, PAI-1, S100A8/A9, sICAM-1, and sP-selectin (Fig. 1 and Table 2).

3.2. Biomarker variation

3.2.1. Control group

The highest CV_I were observed for IL-10, MMP-3, S100/A8/A9 and hs-CRP (Table 3). OxLDL, ADMA, E-selectin, sP-selectin, sVCAM and sICAM-1 demonstrated a CV_I approaching 10%. Between-subject variation was highest for IL-10, hs-CRP and MMP-3 with CV_G above 50%.

An ICC greater than 0.7 was found for hs-CRP, IL-10, oxLDL and sICAM-1, with most other biomarkers having an ICC above 0.5. ADMA, IL-8, PAI-1, S100A8/A9 and sCD40L had an ICC below 0.3. The number of measurements needed to achieve an ICC of 0.75 varied from 1 (hs-CRP, IL-10, sICAM-1 and oxLDL) to up to 13 measurements (S100A8/A9).

3.2.2. PAD group

CV_I in the PAD group was highest for hs-CRP, and low for ADMA, MCP-1, oxLDL, sICAM-1, sP-selectin and sVCAM-1 (Table 4). The highest CV_G was observed for hs-CRP, MMP-3 and IL-10.

With a value of 0.91, D-dimer measurements had the highest ICC, and most biomarkers had ICC's around 0.5 or above. Only S100A8/A9 showed poor reliability with an ICC of 0.27, with 8 measurements required to reach an ICC of 0.75.

3.2.3. PAD group versus control group

Comparing variation between the two groups, CV_I was higher in the PAD group for hs-CRP, IL-6, oxLDL, and sICAM-1 (Tables 3 and 4). Regarding the between-subject variation, CV_G was higher in the PAD group for D-dimer, MMP-9, and PAI-1 (Tables 3 and 4).

4. Discussion

Key findings of our study are that most assessed biomarkers have high within-subject variation both in patients with stable PAD and

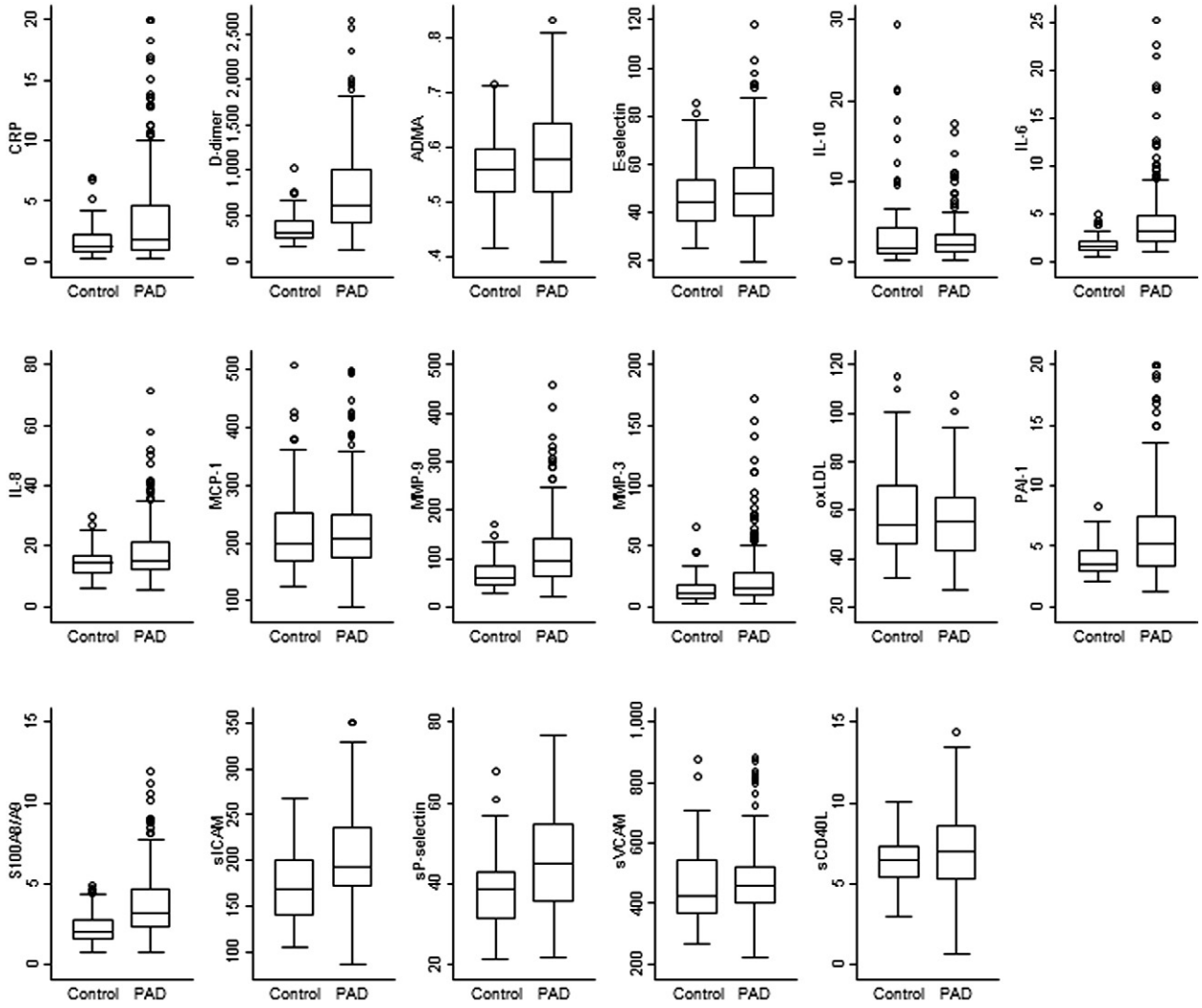

Fig. 1. Box plots of biomarker levels for the control and PAD groups. The boxes represent the lower quartile, the median quartile and the upper quartile. The end of the whiskers indicates the lowest value still within 1.5 times the interquartile range (IQR) of the lower quartile, and the highest value still within 1.5 IQR of the upper quartile.

Table 2
Mean biomarker levels in the control and PAD group.

	Control-group (n = 18)	PAD-group (n = 62)	*P*-value
hs-CRP, mg/L	1.29 (0.93–1.77)	2.09 (1.66–2.63)	0.001
D-dimer, ng/mL	343.8 (295.6–400.0)	626.9 (536.7–732.2)	0.06
ADMA, μmol/L	0.56 (0.54–0.58)	0.57 (0.56–0.59)	0.30
E-selectin, ng/mL	44.1 (39.5–49.3)	48.0 (45.0–51.1)	0.21
IL-10, pg/mL	2.00 (1.20–3.33)	1.91 (1.59–2.30)	0.90
IL-6, pg/mL	1.59 (1.33–1.91)	3.33 (2.95–3.75)	<0.001
IL-8, pg/mL	13.9 (12.5–15.5)	16.2 (14.9–17.8)	0.03
MCP-1, pg/mL	209.6 (185.8–236.5)	212.3 (198.5–227.1)	0.58
MMP-9, ng/mL	62.9 (53.7–73.7)	95.4 (83.9–108.4)	<0.001
MMP-3, ng/mL	11.1 (8.5–14.6)	17.6 (14.8–21.0)	0.05
oxLDL, μU/L	57.9 (50.4–66.6)	53.7 (50.6–57.0)	0.58
PAI-1, ng/mL	3.70 (3.30–4.14)	5.24 (4.53–6.06)	0.002
S100A8/A9, μg/mL	2.11 (1.86–2.39)	3.28 (3.01–3.57)	<0.001
sCD40L, ng/mL	6.45 (5.94–6.96)	6.91 (6.44–7.39)	0.09
sICAM-1, ng/mL	167.3 (152.2–183.8)	197.8 (186.7–209.5)	<.001
sP-selectin, ng/mL	36.9 (33.7–40.5)	42.9 (40.0–46.1)	0.02
sVCAM, ng/mL	443.1 (396.6–495.1)	459.2 (438.4–481.0)	0.91

Note: Data presented as geometric means (95%-confidence intervals, CI), except for sCD40L which showed a normal distribution. The *P*-value for a difference between the groups was calculated from a Wald test of a multilevel mixed-effects linear regression model accounting for the correlation within patients and adjusting for age and gender.

in healthy subjects, suggesting that single measurements provide limited information. However, most biomarkers have moderate to good reliability with repetitive measurements over time making them a potential tool for individual disease monitoring.

Within-subject variation was substantial in most of the assessed biomarkers. The highest CV_I was observed for IL-10 (66.8%) in the healthy control group and for hs-CRP (71.1%) in the PAD group, respectively. Similar data for IL-10 have not yet been published, and reported CV_I for hs-CRP ranged from 42% to 77.7% in healthy subjects, with no specific data on PAD patients [31–33]. Despite its relatively long half-life, factors influencing the high variation of hs-CRP are certainly its important dynamic range as well as its downstream position in the inflammatory cascade [3]. The lowest CV_I with values between 10 and 20% were found for ADMA, E-selectin, MCP-1, oxLDL, sICAM-1, sP-selectin and sVCAM-1 in both groups. For comparison, within-subject variation for lipid parameters is reported to range between 5.8% and 9.3% for total cholesterol [33–35] and 7.8% for LDL-cholesterol according to the database of Dr Ricos, updated in 2014 (accessed at http://www.westgard.com/biodatabase1.htm) [35]. A high within-subject variation reduces the informative value of a single measurement and probably explains numerous inconsistent reports on the association between single biomarker measurement and medical conditions [36,37].

Most studies reporting on repetitive biomarker measurements present ICC values, also known as reliability coefficient calculated

Table 3
Variability components of the different biomarkers in the control group.

Control-group (n = 18)							
	Within-subject CV (%)	Between-subject CV (%)	Intraclass correlation (ICC)	Number of measurements needed*	Index of individuality	Reference change value upwards (%)	Reference change value downwards (%)
hs-CRP	39.0 (29.8 to 46.8)	74.7 (49.9 to 110.7)	0.76 (0.58 to 0.88)	1.0 (0.4 to 2.2)	0.52 (0.32 to 0.84)	183.5 (124.6 to 243.4)	−64.7 (−70.9 to −55.5)
D-dimer	25.2 (20.2 to 30.5)	30.6 (19.2 to 42.9)	0.59 (0.33 to 0.77)	2.1 (0.9 to 6.0)	0.82 (0.53 to 1.43)	98.9 (74.1 to 128.6)	−49.7 (−56.2 to −42.6)
ADMA	11.4 (9.3 to 13.3)	6.2 (2.8 to 9.2)	0.23 (0.05 to 0.43)	10.1 (4.0 to 57.1)	1.84 (1.15 to 4.38)	37.0 (29.3 to 44.3)	−27.0 (−30.7 to −22.7)
E-selectin	19.1 (15.6 to 22.4)	22.2 (17.1 to 27.9)	0.57 (0.41 to 0.73)	2.3 (1.1 to 4.4)	0.86 (0.61 to 1.21)	69.2 (53.6 to 84.6)	−40.9 (−45.8 to −34.9)
IL-10	66.8 (51.9 to 81.0)	128.5 (71.4 to 217.1)	0.73 (0.55 to 0.83)	1.1 (0.6 to 2.4)	0.52 (0.32 to 0.88)	438.1 (287.5 to 615.8)	−81.4 (−86.0 to −74.2)
IL-6	35.1 (28.9 to 41.5)	34.5 (14.4 to 56.3)	0.49 (0.13 to 0.70)	3.1 (1.3 to 19.9)	1.02 (0.64 to 2.64)	157.1 (119.2 to 202.1)	−61.1 (−66.9 to −54.4)
IL-8	29.6 (24.5 to 35.3)	18.4 (5.5 to 29.0)	0.28 (0.03 to 0.55)	7.6 (2.5 to 109.2)	1.61 (0.90 to 6.15)	123.0 (95.5 to 158.6)	−55.2 (−61.3 to −48.8)
MCP-1	20.1 (17.1 to 23.8)	24.2 (11.9 to 35.7)	0.59 (0.23 to 0.78)	2.1 (0.8 to 9.9)	0.83 (0.52 to 1.83)	73.6 (59.9 to 91.5)	−42.4 (−47.8 to −37.5)
MMP-9	33.6 (28.6 to 39.9)	30.1 (21.5 to 39.8)	0.45 (0.28 to 0.62)	3.7 (1.8 to 7.6)	1.12 (0.77 to 1.62)	147.7 (117.4 to 190.2)	−59.6 (−65.5 to −54.0)
MMP-3	51.5 (43.3 to 59.1)	56.4 (38.0 to 78.8)	0.54 (0.34 to 0.70)	2.6 (1.3 to 5.9)	0.91 (0.61 to 1.45)	283.4 (215.3 to 355.5)	−73.9 (−78.0 to −68.3)
oxLDL	11.1 (9.1 to 13.1)	30.1 (22.7 to 38.2)	0.88 (0.81 to 0.92)	0.4 (0.2 to 0.7)	0.37 (0.28 to 0.48)	35.8 (28.8 to 43.6)	−26.4 (−30.4 to −22.3)
PAI-1	29.4 (25.8 to 32.8)	18.0 (9.9 to 26.6)	0.28 (0.09 to 0.47)	7.8 (3.4 to 29.9)	1.63 (1.07 to 3.23)	122.4 (102.1 to 142.3)	−55.0 (−58.7 to −50.5)
S100A8/A9	39.7 (33.7 to 45.0)	18.7 (6.9 to 29.7)	0.19 (0.03 to 0.40)	12.8 (4.5 to 110.8)	2.12 (1.23 to 6.32)	188.5 (147.9 to 228.9)	−65.3 (−69.6 to −59.7)
sICAM-1	10.4 (8.5 to 12.0)	19.3 (15.4 to 24.6)	0.77 (0.67 to 0.86)	0.9 (0.5 to 1.5)	0.54 (0.41 to 0.71)	33.2 (26.7 to 39.1)	−24.9 (−28.1 to −21.1)
sP-selectin	14.7 (11.8 to 17.5)	18.6 (14.4 to 24.0)	0.61 (0.50 to 0.70)	1.9 (1.3 to 3.0)	0.79 (0.64 to 1.00)	50.1 (38.4 to 61.8)	−33.4 (−38.2 to −27.7)
sVCAM	18.1 (13.1 to 23.0)	22.3 (15.3 to 30.5)	0.60 (0.35 to 0.81)	2.0 (0.7 to 5.6)	0.81 (0.47 to 1.37)	64.5 (43.5 to 87.6)	−39.2 (−46.7 to −30.3)
sCD40L	20.4 (16.7 to 23.8)	13.2 (6.9 to 19.3)	0.29 (0.10 to 0.49)	7.2 (3.2 to 26.3)	1.55 (1.03 to 2.96)	56.5 (46.3 to 66.0)	−56.5 (−66.0 to −46.3)

Note: CV, coefficient of variation; * the number of measurements needed to achieve an reliability (ICC) of 0.75; for practical use this number needs to be rounded off to a whole number ≥ 1.

as the ratio of the between-subject variance to the total variance [38–40]. Expressed differently, an ICC close to 1 signifies little variation within compared to the variation between subjects [41]. The ICCs reported here show a wide range from 0.19 for S100A8/A9 to 0.88 for oxLDL in healthy controls, with similar values found in PAD patients. The majority of biomarkers tested have an ICC of more than 0.5, suggesting moderate to good reliability for repetitive individual measurements over time. Based on our results the best candidates for individual follow-up are hs-CRP, D-Dimer, E-selectin, IL-10, MCP-1, MMP-3, oxLDL, sICAM-1, sP-selectin and sVCAM in healthy controls; and hs-CRP, D-dimer, E-selectin, IL-10, MCP-1, MMP-9, MMP-3, oxLDL, PAI-1, sCD40L, sICAM and sP-selectin in PAD patients. The interpretation of our results becomes more obvious when compared to the variation of some of the most commonly assessed parameters in cardiovascular medicine. The ICC for systolic and diastolic blood pressure has recently been reported in 2 large population-based cohort studies to be between 0.57 to 0.65 and 0.57 to 0.70, respectively [40,42]. The ICC of total cholesterol is generally reported to be higher with values between 0.62 and 0.82 [39,40,43]. Glynn and colleagues found an ICC for LDL-cholesterol of 0.56, similar to most of the analyzed biomarkers of the present study [39].

An option to increase the reliability of a biomarker is to calculate the mean of repetitive measurements. In order to achieve the somewhat arbitrarily chosen good reliability ICC threshold of 0.75, as reported by Koenig and coworkers for total cholesterol as reference for cardiovascular risk assessment [40], most biomarkers evaluated in the present study require a mean of 1 to 3 measurements. For hs-CRP, our results are in line with other groups that proposed to use 2 to 3 consecutive measurements [37,40,43]. Underlining this concept, Sapey and coworkers demonstrated that taking a 3-day mean of inflammatory biomarkers in a sputum sample in patients with chronic obstructive pulmonary disease significantly improved reliability by reducing the within-subject variation without altering the between-subject variation. Thereby a dramatic reduction of the needed sample size for interventional studies could be achieved [44]. Similarly, Danesh and coworkers showed that the odds ratio for developing coronary heart disease increased from 1.46 (95%-CI 1.29–1.65) for baseline IL-6 levels to 2.14 (95%-CI 1.45–3.15) after correction for within-subject variation

Table 4
Variability components of the different biomarkers in the PAD group.

PAD-group (n = 62)							
	Within-subject CV (%)	Between-subject CV (%)	Intraclass correlation (ICC)	Number of measurements needed*	Index of individuality	Reference change value upwards (%)	Reference change value downwards (%)
hs-CRP	71.1 (57.3 to 87.4)	105.4 (79.4 to 135.8)	0.65 (0.49 to 0.77)	1.6 (0.9 to 3.2)	0.67 (0.46 to 1.04)	488.9 (338.1 to 706.8)	−83.0 (−87.6 to −77.2)
D-dimer	19.9 (17.0 to 23.3)	67.8 (55.4 to 82.3)	0.91 (0.86 to 0.94)	0.3 (0.2 to 0.5)	0.29 (0.22 to 0.39)	72.8 (59.7 to 89.0)	−42.1 (−47.1 to −37.4)
ADMA	12.3 (11.0 to 13.8)	9.3 (7.0 to 11.7)	0.36 (0.22 to 0.51)	5.2 (2.9 to 10.8)	1.32 (0.98 to 1.91)	40.5 (35.4 to 46.2)	−28.8 (−31.6 to −26.2)
E-selectin	20.7 (18.7 to 22.9)	23.1 (19.1 to 27.8)	0.55 (0.45 to 0.65)	2.4 (1.6 to 3.6)	0.90 (0.73 to 1.10)	76.5 (67.2 to 87.0)	−43.3 (−46.5 to −40.2)
IL-10	64.8 (55.2 to 76.1)	75.3 (49.8 to 104.0)	0.56 (0.36 to 0.71)	2.3 (1.2 to 5.3)	0.86 (0.58 to 1.39)	415.7 (317.6 to 551.3)	−80.6 (−84.6 to −76.1)
IL-6	46.8 (40.5 to 54.4)	43.6 (33.6 to 55.8)	0.47 (0.33 to 0.60)	3.4 (2.0 to 6.2)	1.07 (0.80 to 1.48)	243.3 (194.4 to 310.0)	−70.9 (−75.6 to −66.0)
IL-8	35.0 (31.9 to 38.4)	32.0 (23.9 to 41.2)	0.46 (0.31 to 0.59)	3.6 (2.1 to 6.6)	1.10 (0.82 to 1.51)	156.7 (136.7 to 179.6)	−61.1 (−64.2 to −57.8)
MCP-1	18.9 (16.7 to 21.2)	25.3 (19.0 to 32.4)	0.64 (0.49 to 0.76)	1.7 (1.0 to 3.1)	0.75 (0.56 to 1.02)	68.1 (58.3 to 78.9)	−40.5 (−44.1 to −36.8)
MMP-9	37.9 (34.0 to 42.3)	50.6 (41.2 to 60.9)	0.63 (0.52 to 0.72)	1.8 (1.2 to 2.8)	0.75 (0.60 to 0.96)	175.9 (150.0 to 207.7)	−63.8 (−67.5 to −60.0)
MMP-3	50.8 (45.0 to 56.8)	72.2 (54.5 to 94.4)	0.65 (0.51 to 0.76)	1.6 (1.0 to 2.9)	0.70 (0.51 to 0.98)	277.2 (229.1 to 333.3)	−73.5 (−76.9 to −69.6)
oxLDL	17.8 (15.7 to 19.9)	22.2 (17.7 to 27.0)	0.61 (0.47 to 0.72)	2.0 (1.2 to 3.4)	0.80 (0.61 to 1.07)	63.3 (54.3 to 72.8)	−38.8 (−42.1 to −35.2)
PAI-1	32.4 (28.1 to 37.1)	60.4 (49.0 to 73.0)	0.76 (0.66 to 0.83)	1.0 (0.6 to 1.5)	0.54 (0.42 to 0.69)	139.9 (114.8 to 170.8)	−58.3 (−63.1 to −53.4)
S100A8/A9	44.8 (40.1 to 49.9)	26.4 (18.6 to 34.8)	0.27 (0.15 to 0.40)	8.2 (4.4 to 17.0)	1.70 (1.24 to 2.48)	227.4 (191.6 to 269.7)	−69.5 (−73.0 to −65.7)
sICAM-1	13.7 (12.0 to 15.4)	22.2 (17.7 to 27.0)	0.72 (0.61 to 0.81)	1.2 (0.7 to 2.0)	0.62 (0.48 to 0.81)	45.8 (39.3 to 52.9)	−31.4 (−34.6 to −28.2)
sP-selectin	13.9 (12.3 to 15.8)	27.2 (22.9 to 31.9)	0.79 (0.70 to 0.85)	0.8 (0.5 to 1.3)	0.51 (0.41 to 0.65)	46.9 (40.4 to 54.7)	−31.9 (−35.4 to −28.8)
sVCAM	17.9 (15.3 to 20.4)	16.2 (12.9 to 19.7)	0.45 (0.34 to 0.55)	3.6 (2.4 to 5.9)	1.10 (0.89 to 1.40)	63.5 (52.5 to 75.2)	−38.8 (−42.9 to −34.4)
sCD40L	24.1 (21.4 to 27.0)	24.0 (17.4 to 31.5)	0.50 (0.33 to 0.64)	3.0 (1.7 to 6.1)	1.01 (0.75 to 1.43)	66.8 (59.3 to 74.9)	−66.8 (−74.9 to −59.3)

Note: CV, coefficient of variation; * the number of measurements needed to achieve a reliability (ICC) of 0.75; for practical use this number needs to be rounded off to a whole number ≥ 1.

by taking paired IL-6 measurements on average 4 years apart [45]. According to our presented data, 3 measurements of IL-6 would have been required to reach an ICC of 75%, potentially further increasing the association between IL-6 levels and coronary heart disease.

Higher mean levels of hs-CRP, D-dimers, IL-6, MMP-9, PAI-1, sICAM and sP-selectin in patients with atherosclerosis as reported in our study have already been found in several cross-sectional studies [11–13,46,47]. In contrast to other authors, we did not find increased levels of ADMA [48], MCP-1 [49], oxLDL [50], or sCD40 [51]. Potential explanations for this difference might be our longitudinal study design including 4 measurements per participant or the fact that disease severity and stability differed between these studies. The new finding is that IL-8, a chemokine [3], and S100A8/A9, a member of the S100 calcium-binding family of proteins with complex immune regulatory actions are increased in patients with PAD [3,52]. Both IL-8 and in particular S100A8/A9, also known as myeloid-related protein (MRP)-8/14, have recently gained much interest in cardiovascular research [52–54]. Nevertheless, due to the high within-subject variation and the low ICC even in stable PAD patients, practical use of S100A8A as a serum biomarker and to a lesser extent of IL-8 is compromised.

Among the biomarkers with increased mean values in patients with PAD, hs-CRP, IL-6, and sICAM-1 also showed a higher within-subject variation than in the control group. A pathophysiological explanation for this observation could be subclinical inflammatory bouts as a marker of plaque instability [3]. Interestingly, Berger and coworkers reported in a cohort of patients with coronary artery disease or its risk equivalent, that those patients with concomitant PAD had higher levels of hs-CRP, IL-6, MMP-9, ICAM-1 than patients without PAD [15]. These biomarkers are likely to be of particular importance in the pathophysiology of atherosclerotic PAD and might therefore be of particular interest for diagnosis, subclinical disease monitoring or prognostic stratification [24,55]. In contrast to our results, de Maat and coworkers reported no difference in within-subject- and between-subject variation of CRP and PAI-I between a group of healthy young adults versus patients with clinically stable angina pectoris [56]. These conflicting results might be explained by the higher inflammatory activity in patients with PAD than in patients with coronary disease as reported by Berger and coworkers [15].

4.1. Study limitations

Our study has several limitations. First, many baseline characteristics differ between the control and PAD group. Although we adjusted for differences in age and gender when comparing the mean biomarker levels of both groups patients often have other co-morbidities influencing results. Second, the study groups, especially the control group, were rather small. Nevertheless, most other studies analyzing the variation components of biomarkers in patients and healthy controls reported results based on similar sample sizes [32,56,57]. Third, we could not assess analytic variation. Total within-subject biological variation is considered to be the sum of analytic variation and biologic within-subject variation [21]. Because of the multitude of biomarkers assessed in our study, each biomarker was only analyzed once to limit not only the study costs but also the quantity of blood needed per patient at each visit.

5. Conclusions

In summary, the knowledge of a biomarker's variation is crucial for its correct interpretation. This study presents the variation of established and emerging serum biomarkers of atherosclerosis, both for patients with clinically manifest atherosclerosis and for healthy controls. Our results suggest that for most of the analyzed biomarkers single measurements are of little value, and that with single measurements relevant associations between biomarkers and disease states could be missed. However, due to the moderate to good reliability with repetitive measurements over time, most biomarkers are suitable for individual follow up and disease monitoring. Among the biomarkers with increased mean values in patients with PAD, the best variation profile was found for hs-CRP, MMP-9, MMP-3, D-dimer, PAI-1, sP-selectin and sICAM-1. These biomarkers deserve special attention in future studies. In order to further define the place of biomarkers in clinical practice, advanced phase biomarker studies such as decision-analysis modeling are needed [18]. The presented results are a helpful source of information for the design of these studies.

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