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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.

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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

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