Supplementary material

Sphingosine 1-phosphate (S1P) levels in plasma and HDL are altered in coronary artery disease

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Methods

Study participants

From 2006 to 2007, blood samples were prospectively taken from consecutive patients ≥18 years of age presenting either with acute myocardial infarction (MI, ST-segment elevation myocardial infarction [1] or non-ST-segment elevation MI [2]) or with stable coronary artery disease (sCAD) at the Clinic of Cardiology, West German Heart Center, University Hospital Essen (patients with MI and with sCAD) and the Alfried Krupp Hospital Essen (patients with MI). Healthy, unrelated blood-donors from the Institute for Transfusion Medicine, University of Essen School of Medicine, were invited to participate as controls (control group). All blood-donors were in normal health, based on cardiovascular and other medical history, physical examination, and biochemical and hematological screening, and without signs and symptoms of coronary artery disease.

Blood Collection and Plasma Preparation

In the MI group, blood sampling was performed during percutaneous coronary artery intervention for the treatment of the myocardial infarction as soon as the patient was clinically stabilized via the inserted arterial sheath or via an inserted venous catheter. In the sCAD group, study samples were collected during a routine blood sampling from peripheral veins. If a percutaneous coronary angiography had been performed in this study group for disease evaluation, the blood collection was undertaken on the day following the angiography to rule out an acute phase reaction upon vascular manipulation. In the control group, blood was drawn directly after blood donation through the same venipuncture set used for the donation process. In all groups, 30 ml of blood was drawn into vacuum tubes containing 1.6mg EDTA/mL (4.298mM EDTA/L) to prevent release of S1P from blood cells during clot formation. Immediately after blood drawing, the vacuum tubes were placed on ice and stored
at 4°C until further processing. Plasma was generated by centrifugation (3000 rpm, 30 minutes, 4°C), immediately recovered and frozen at -80°C.

**Determination of Plasma and HDL parameters**

Levels of total cholesterol, LDL-C and HDL-C of the obtained plasma and cholesterol levels of isolated HDL were determined in the central laboratory of the University Hospital Essen by enzymatic methods (TC: Advia Chemistry Systems, coefficient of variance (CV) 1.45%; LDL-C: Advia Chemistry Systems DLDL, CV 2.45%; HDL-C: Advia Chemistry Systems DHDL, CV 2.36%; all test were from Bayer Health Care, Germany).

**Isolation of High-Density Lipoprotein**

All experimental procedures were performed by an investigator blinded to patients’ data. High-density lipoproteins were isolated by sequential density gradient ultracentrifugation according to their density (1.069–1.21 g/mL [3]), following an established protocol [4]. Protein concentration was determined in each sample by Bradford assay (Bio-Rad, USA).

**Determination of Sphingosine-1-Phosphate**

Determination of Sphingosine-1-Phosphate was performed according to an established protocol by HPLC analysis [5]. Briefly, lipids were extracted from the HDL samples by successive addition of 1mL of methanol, 200µl of 6M HCl and twice 2mL of chloroform. Chloroform phases were retrieved by centrifugation, and chloroform was removed by vacuum-drying in a speed-vac. Subsequently, samples were dissolved in 200µl of dioxane, 200µl of 70mM K_2HPO_4 and 200µl of 9-fluorenylmethyl chloroformate (FMOC-Cl) solution for derivatization of sphingolipids. For chromatographic detection of sphingolipids (Merck-Hitachi Elite LaChrom System, VWR, Darmstadt, Germany), 10µl of sample was injected by cut-injection method with an injection-pump delivery rate of 1.3 ml/min into an eluent
containing methanol, 70mM K$_2$HPO$_4$, and H$_2$O. Columns used for the separation of sphingolipids by reversed phase HPLC were a 250 × 4.6 mm Kromasil 100-5 C18 column and a 17 × 4 mm Kromasil 100-5 C18 pre-column (CS Chromatographie Service, Langerwehe, Germany) set to 35 °C. Detection was performed with a fluorescence detector (excitation 263nm, emission 316nm). Loss of HDL during ultracentrifugation has been described previously [3]. To account for this fact, the values of HDL-bound plasma S1P [pmol/mL] were multiplied with a correction factor which accounts for losses of HDL by equalizing the cholesterol amount present in the plasma used for isolation with the factually measured cholesterol in the isolated HDL (total HDL-C amount of the plasma volume used ([mg], derived by multiplying the plasma HDL-C concentration [mg/mL] with the plasma volume used [mL]), divided by the total cholesterol amount in the isolated HDL ([mg], derived by multiplying the cholesterol concentration of the isolated HDL [mg/mL] with the final volume of the isolate [mL])).

**Statistical Methods**

Data are expressed by mean ± standard deviation or median (range) for continuous variables, and frequency count and percentage for qualitative variables. All statistical analyses were performed by K. Sattler. Mann-Whitney-rank-sum test, Kruskal-Wallis test, or Wilcoxon test for paired non-parametric data were used for the comparison of groups. The association of variables were tested with Spearman’s rank-order correlation followed by linear regression analysis. One outlier in both the sCAD and the control group was excluded from the test of correlation for “plasma S1P” and “ratio of non-HDL-bound plasma S1P to normalized HDL-bound plasma S1P”. A multivariable linear regression analysis utilizing single-step block entry of the predictor variables was performed with the dependent variables "plasma S1P”, “non-HDL-bound plasma S1P” and “HDL-bound plasma-S1P” and the predictor variables “plasma HDL-C levels, age, plasma LDL-C, intake of statins (MI and sCAD group only)".
To analyze the influence of demographic, clinical or pharmacologic variables on S1P, the parameter “non-HDL-bound plasma S1P” was adjusted for the variables “glomerular filtration rate ≥60ml/min”, “current smoking”, the intake of the drugs listed in table 3, and the polytomous variables “extent of disease” and “CCS 0-IV” in both patient groups. In the sCAD group, adjustment was made additionally for the variable “invasive re-evaluation of CAD” and in the MI group for the variables “ST-segment elevation myocardial infarction” and “intake of cardiovascular medication prior to infarction”. Influence of pharmacologic treatment was further tested by comparing S1P parameters of MI patients taking cardiovascular medication prior to MI with patients with sCAD, and by comparing S1P parameters of MI patients not taking cardiovascular medication prior to MI with controls. Receiver-operating characteristic (ROC) curves and area under the curve (AUC, assumed non-parametric distribution) were calculated to evaluate the parameters “total plasma S1P”, “non-HDL-bound plasma S1P”, and “ratio of non-HDL-bound plasma S1P to normalized HDL-bound plasma S1P” to discriminate between patients with MI or with sCAD and controls. Results presented are the calculated value for the AUC, the standard error, the P value, and the 95% confidence interval. P-values are understood to be strictly descriptive. Statistical significance was assumed for P<0.05. All analyses and graphs were performed with SPSS 17.0 (Chicago, USA).
Results

Among the study groups, the correction factors as defined in the methods section were comparable (MI: 3.69 [1.14-9.43], sCAD: 3.73 [2.24-39.12], controls: 3.40 (1.48-15.57), P=0.29).

The content of protein or cholesterol in HDL was positively associated with the HDL content of S1P (MI: HDL protein and HDL S1P: r=0.59, β=0.56, r²=0.31, P<0.001, 95% CI 103.35-205.64, HDL cholesterol and HDL S1P: r=0.70, β=0.69, r²=0.48, P<0.001, 95% CI 350.27-558.57; sCAD: HDL protein and HDL S1P: r=0.54, β=0.55, r²=0.30, P<0.001, 95% CI 77.24-18.81, HDL cholesterol and HDL S1P: r=0.65, β=0.59, r²=0.35, P<0.001, 95% CI 197.53-352.62; controls: HDL protein and HDL S1P: r=0.76, β=0.75, r²=0.57, P<0.001, 95% CI 145.90-214.56, HDL cholesterol and HDL S1P: r=0.77, β=0.76, r²=0.57, P<0.001, 95% CI 343.43-503.26). Levels of total plasma S1P were positively associated with the S1P content of isolated HDL in each study group as well (MI: S1P/protein: r=0.56, β=0.57, r²=0.32, P<0.001, 95% CI 0.18-0.35, S1P/cholesterol: r=0.42, β=0.41, r²=0.17, P<0.001, 95% CI 0.33-0.96; sCAD: S1P/protein: r=0.45, β=0.32, r²=0.11, P=0.001, 95% CI 0.04-0.17, S1P/cholesterol: r=0.38, β=0.24, r²=0.06, P=0.019, 95% CI 0.05-0.53; controls: S1P/protein: r=0.34, β=0.33, r²=0.11, P=0.002, 95% CI 0.06-0.24, S1P/cholesterol: r=0.33, β=0.38, r²=0.14, P<0.001, 95% CI 0.27-0.89).

The S1P content of isolated HDL relative to their protein or cholesterol content was positively associated with the plasma levels of HDL-bound S1P (MI: S1P/protein: r=0.83, β=0.80, r²=0.64, P<0.001, 95% CI 1.16-1.63, S1P/cholesterol: r=0.76, β=0.75, r²=0.56, P<0.001, 95% CI 0.31-0.47, S1P/cholesterol:protein: β=0.67, r²=0.45, P<0.001, 95% CI 0.11-0.19; sCAD: S1P/protein: r=0.76, β=0.75, r²=0.56, P<0.001, 95% CI 0.99-1.43, S1P/cholesterol: r=0.82, β=0.76, r²=0.60, P<0.001, 95% CI 0.29-0.41, S1P/cholesterol:protein: β=0.75, r²=0.57, P<0.001, 95% CI 0.12-0.17; controls: S1P/protein: r=0.76, β=0.71, r²=0.51, P<0.001, 95% CI
1.29-2.00, S1P/cholesterol: \( r=0.67, \beta=0.64, r^2=0.41, P<0.001, 95\% CI 0.32-0.55, \) 
S1P/cholesterol:protein: \( \beta=0.70, r^2=0.49, P<0.001, 95\% CI 0.01-0.18). \)

Plasma HDL-C levels predicted levels of HDL-bound plasma S1P in the multiple linear regression analysis in each study group (MI: \( \beta=0.67, r^2=0.45, P<0.001, 95\% CI \) (confidence interval) 425.88, 813.14, sCAD: \( \beta=0.47, r^2=0.22, P<0.001, 95\% CI 226.39, 552.39, \) controls: \( \beta=0.42, r^2=0.19, P<0.001, 95\% CI 145.71, 443.95). Levels of plasma S1P or non-HDL-bound plasma S1P were not predicted by any of the variables included in the regression model in any of the study groups (data not shown).

Demographic, clinical or pharmacologic variables did not show an influence of any of the tested variables (described in detail in the methodology paragraph) on the levels of normalized non-HDL-bound plasma S1P, except as follows: Only in sCAD patients, there were differences between male and female participants in that male patients had lower values for HDL-bound plasma S1P but higher values for non-HDL-bound plasma S1P and derived parameters than female patients (HDL-bound plasma S1P in male patients: 191.05pmol/mL [46.67-555.45] vs. 233.68pmol/mL [96.25-429.48] in female patients, \( P=0.02, \) non-HDL-bound plasma S1P in male patients: 108.39pmol/mL [0.00-1065.94] vs. 11.5pmol/mL [0.00-821.32] in female patients, \( P=0.001, \) fraction of non-HDL-bound plasma S1P in male patients: 41.75% [0.00-87.00] vs. 3.52% [0.00-0.78] in female patients, \( P<0.001, \) ratio of non-HDL-bound plasma S1P/normalized HDL-bound plasma S1P in male patients 0.24mg/mL [0.00-2.52 vs. 0.02mg/mL [0.00-1.62] in female patients, \( P=0.002). \)

Normalized HDL-bound plasma S1P was not different between male and female patients (470.96pmol/mg [166.67-1400.00] vs. 451.28pmol/mg [250.00-816.67] in female patients, \( P=0.90, \) These findings were not due to lower levels of plasma HDL-C in male than in female patients with sCAD (0.40mg/mL [0.23-0.69] vs. 0.51mg/mL [0.28-0.88], \( P<0.001) \) as in both other study groups male participants had lower levels of plasma HDL-C than female
participants (MI: 0.38mg/mL [0.07-0.93] vs. 0.48mg/mL [0.21-0.80], P=0.01; controls: 0.57mg/mL [0.29-1.14] vs. 0.68mg/mL[0.42-1.11], P=0.005).

To look deeper into a potential influence of gender on the results, we compared male and female patients of the study groups separately. Comparison of male participants showed the same results as the overall analyses except for the level of normalized HDL-bound plasma S1P, which was higher in MI than in sCAD (MI, n=52: 541.48pmol/mg [217.14-1529.41], sCAD, n=70: 470pmol/mg [166.67-1400.00], controls, n=45: 518.52pmol/mg [252.00-891.23], MI vs. sCAD: P=0.036, controls vs. each patient group: P>0.05). Comparison of female participants across groups showed the same differences as in the overall analysis when comparing patients with MI with controls, but no differences between sCAD and controls (except of HDL-bound plasma S1P), and none between sCAD and MI (data not shown). Thus, the results of the overall analysis were driven by the results of the male patients in the sCAD group, whereas in both other groups the gender did not influence the results.

When combining the patients’ groups according to symptoms, the levels of non-HDL-bound plasma S1P increased with increasing severity of angina pectoris as defined by the Canadian Cardiovascular Score (CCS; CCS 0, control group: 10.93pmol/mL [0.00-369.54], CCS I: 72.03pmol/mL [0.00-1065.94], CCS II: 85.00pmol/mL [0.00-821.32], CCS III: 195.51pmol/mL [0.00-334.05], CCS IV, MI group: 78.69pmol/mL [0.00-618.39]; CCS 0 vs. CCS I: P=0.013, CCS 0 vs. CCS II: P=0.002, CCS 0 vs. CCS III: P=0.011, CCS 0 vs. CCS IV: P<0.001, all other comparisons: P>0.05). The levels of normalized HDL-bound plasma S1P were: CCS 0, control group: 522.73pmol/mg [30.77-1043.14], CCS I: 439.80pmol/mg [210.00-1096.30], CCS II: 423.08pmol/mg [166.67-1400.00], CCS III: 546.48pmol/mg [357.14-1037.50], CCS IV, MI group: 524.14pmol/mg [202.13-1529.41]; CCS 0 vs. CCS I: P=0.039, CCS 0 vs. CCS II: P=0.026, CCS I vs. CCS IV: P=0.039, CCS II vs. CCS IV: P=0.028, all other comparisons: P>0.05.
To estimate a potential influence of pharmacologic treatment of CAD, the analyses were repeated by comparing the patients with sCAD with MI that were taking cardiovascular medication prior to myocardial infarction (Table 3). MI patients had higher levels of normalized HDL-bound plasma S1P than patients with sCAD (507.87pmol/mg [202.13-1529.41] vs. 465.00pmol/mg [166.67-1400.00], P=0.036), while the other parameters were not different between the groups. The comparison of the controls with patients with MI not taking cardiovascular medication (Table 3) prior to myocardial infarction showed similar results as obtained in the overall analyses (data not shown). Only in the MI group, a subgroup of patients taking statins was comparable to a subgroup not taking statins due to the small size of the group of patients not taking statins in the sCAD group. There were no differences regarding the S1P-parameter between patients taking and not taking statins prior to myocardial infarction (data not shown). During PCI, additional treatment with abciximab was considered necessary in several of the MI patients (n=27), but unnecessary in others (n=45). There were no differences in plasma S1P levels between patients treated and patients not treated with abciximab (310pmol/mL [160.00-759.92] vs. 348.00pmol/mL [125.00-853.25], P=0.91). This is in line with a previous study, in which no influence of the administration of abciximab on the levels of markers of inflammation, coagulation and fibrinolysis was observed [6].
References


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<th>MI (n=83)</th>
<th>Stable CAD (n=95)</th>
<th>P value</th>
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<tr>
<td>Cardiovascular medication prior to MI — no. (%)</td>
<td>50/74 (67.6)</td>
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<td>Acetylsalicylate — no. (%)</td>
<td>33/68 (48.5)</td>
<td>88 (94.6)</td>
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<td>Beta-Blockers — no. (%)</td>
<td>37/68 (54.4)</td>
<td>86 (92.5)</td>
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<td>ACE-inhibitors — no. (%)</td>
<td>26/68 (38.2)</td>
<td>58 (62.4)</td>
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<td>AT1-blockers — no. (%)</td>
<td>8/68 (11.8)</td>
<td>26 (28.0)</td>
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<td>Diuretics — no. (%)</td>
<td>22/68 (32.4)</td>
<td>66 (71.0)</td>
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<td>Calcium channel-blockers — no. (%)</td>
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<td>21 (22.6)</td>
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<td>16/68 (23.5)</td>
<td>41 (44.1)</td>
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<td>Phenprocoumon — no. (%)</td>
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<td>12 (12.9)</td>
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<td>Digitalis — no. (%)</td>
<td>2/68 (2.9)</td>
<td>6 (6.5)</td>
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Supplemental table. Medication of the patient population at time of enrolment. *

* Values given are numbers and percentages.
For seven patients, specification of drug intake prior to MI was not available. Thus data are given as number of positive observations/total recorded counts of observations (percent of total recorded counts of observation).

Abbreviations: ACE-inhibitors, angiotensin converting enzyme-inhibitors; AT1-blockers, angiotensin receptor type I-blockers; MI, acute myocardial infarction.