Hypertension

ORIGINAL ARTICLE

Plasma S1P (Sphingosine-1-Phosphate) Links to Hypertension and Biomarkers of Inflammation and Cardiovascular Disease

Findings From a Translational Investigation

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ABSTRACT: S1P (Sphingosine-1-phosphate) is an important regulator of immune cell trafficking and vascular dysfunction contributing to the development and progression of overt hypertension. Although targeting S1P signaling revealed therapeutic potential in different experimental hypertension studies, validations of S1P-blood pressure (BP) associations in humans are lacking. In a translational approach, we explored the associations between plasma S1P and BP in a family based study cohort (MOS [Malmö Offspring Study]; N=1046) and in a longitudinally conducted murine hypertension cohort. In MOS, linear multivariate regression analyses showed that plasma S1P associates with increased systolic BP (β=1.06, P=0.015). Study subjects with systolic BP ≥140 mm Hg presented with significantly higher S1P plasma concentrations compared with subjects with BP <120 mm Hg independent of age and sex. The S1P-BP association was validated in a murine model where plasma S1P increased with systolic BP (r=0.7018, R^2=0.4925; P<0.0001). In a subsample of MOS (N=444), proteomic profiling for markers of inflammation, metabolism, and cardiovascular disease using Proximity Extension Assays revealed multiple significant S1P associations, some of them with marked sex-specificity. In vitro and ex vivo validation of identified S1P associations disclosed augmented expression of different vascular dysfunction and inflammation markers in response to S1P. Our translational findings show a link between plasma S1P and systolic BP as well as several inflammation and cardiovascular disease markers and suggest S1P's biomarker potential. This encourages further studies to investigate its predictive capacity for hypertensive disease or the therapeutic potential of its signaling axis. **(*Hypertension*. 2021;78:00–00. DOI: 10.1161/HYPERTENSIONAHA.120.17379.)** • **Data Supplement**

Key Words: biomarkers ■ blood pressure ■ cardiovascular disease ■ inflammation ■ sphingosine-1-phosphate

Hypertension is the most common preventable risk factor for cardiovascular disease (CVD) and leading single contributor to all-cause mortality and disability worldwide.[1] Blood pressure (BP) control is, therefore, considered the gold standard approach to reducing the proportion of population burden of BP-induced CVD. The existing link between BP and cardiovascular risk[1,2] highlights the need to intervene early. Following the results of the SPRINT study (Systolic Blood Pressure Intervention Trial),[3] both the American College of Cardiology/American Heart Association and the European Society of Cardiology/European Society of Hypertension have gradually updated their advice on diagnosis and treatment of arterial hypertension.[4,5] The implementation of lower BP thresholds[5] ultimately increases the prevalence of hypertension substantially,[6] with a large young low-risk population who are recommended antihypertensive treatment (AHT) according to the new guidelines. Undoubtedly, this would result in treatment of a substantial amount of people with no or

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ORIGINAL ARTICLE

Novelty and Significance

What Is New?

- First study to assess plasma S1P (sphingosine-1-phosphate) levels in a large family based study cohort (N=1046).
- Proteomic profiling that revealed associations between plasma S1P and multiple cardiovascular, inflammation, and metabolism biomarkers.

What Is Relevant?

- S1P levels increase with systolic blood pressure.
- Blood pressure but not plasma S1P differs sex specifically.
- Sex-specific S1P associations with several biomarkers, including IL (interleukin)-18 exist but are mostly blood pressure-independent.

Summary

For the first time, it is shown that plasma S1P levels increase with systolic blood pressure in a large relatively young and healthy population cohort where it also associates with several cardiovascular, inflammation, and metabolism biomarkers.

Nonstandard Abbreviations and Acronyms

AHT	antihypertensive treatment
Ang II	angiotensin II
Ang-1	angiopoietin 1
BMI	body mass index
BP	blood pressure
CVD	cardiovascular disease
eGFR	estimated glomerular filtration rate
IL	interleukin
MDCS-CC	Malmö Diet and Cancer Study—Cardiovascular Cohort
MOS	Malmö Offspring Study
PAI	plasminogen activation inhibitor 1
PECAM-1	platelet endothelial cell adhesion molecule 1
SELP	P-selectin
SphK2	sphingosine kinase 2
SPRINT	Systolic Blood Pressure Intervention Trial
ST2	soluble interleukin 1 receptor-like 1
t-SNE	t-distributed stochastic neighbor embedding

little benefit. Thus, the incorporation of biomarkers to aid in the selection of patients that would actually respond to antihypertensive therapy (personalized medicine)[7] and for the overall assessment of cardiovascular risk has been proposed.[8–11] Selecting from the numerous inflammation, metabolism, and CVD biomarkers that associate with a higher cardiovascular risk might inflict bias, hence, the use of biomarkers with association to both BP and established CVD, inflammation, and metabolism markers might be advantageous.

As major regulator of vascular functions, inflammatory, and metabolic processes relevant to the pathology of hypertension and associated cardiovascular events the bioactive sphingophospholipid S1P (sphingosine-1-phosphate) might possess such biomarker potential.[12–18] Besides holding cell type- and receptor-specific vasomodulator potency,[12,14] S1P signaling critically regulates important immune system functions,[19–22] is involved in barrier function control,[23,24] and largely affects lipid and glucose metabolism.[25–27] In experimental studies, augmented S1P signaling has been linked to several cardiovascular condition, including stroke, heart failure, atherosclerosis, and hypertension.[12,13,15,28] Small scale biomarker studies associated plasma S1P levels with impaired left ventricular ejection fraction (N=74) and all-cause mortality (N=210) in systolic heart failure patients.[29,30] Plasma S1P was further used to predict the severity of coronary artery atherosclerosis (N=59).[31] With respect to hypertension, S1P's role in disease development and propagation is mainly elusive,[13] despite a few reports that indicate altered sphingolipid metabolism in different forms of experimental hypertension.[15,28,32–35] To date, human cohort-based studies investigating associations between S1P plasma concentrations and BP are lacking. Therefore, this study explored the relationship between plasma S1P and BP in a large family based cohort study, clinically validating results obtained in an experimental murine hypertension model. Additionally, evaluation of S1P associations with established CVD, inflammation, and metabolism biomarkers using multiplex proteomic profiling and in vitro and ex vivo validation approaches were performed to study novel associations with established biomarkers relevant to hypertensive disease.

METHODS

The data underlying this article are available in the article and in its Data Supplement. Study population-specific data that support the findings of this study are available upon reasonable request from the Steering Committee of Malmö Diet and Cancer study by contacting its chair, Professor Olle Melander

(olle.melander@med.lu.se) or Professor Peter M. Nilsson (peter.nilsson@med.lu.se), but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available due to ethical and legal restrictions related to the Swedish Biobanks in Medical Care Act (2002:297) and the General Data Protection Regulation (GDPR) Act (2016/679) as well as related regulations.

Human Study Population

The MOS (Malmö Offspring Study) is an ongoing family based cohort study.[36] The study population consists of adult children (MOS-G2) and grandchildren (MOS-G3) of participants from the MDCS-CC (Malmö Diet and Cancer Study—Cardiovascular Cohort).[37] Participants were recruited using official register information from the Swedish Tax Agency. Not understanding information in Swedish was the only exclusion criteria. The study population used in the current investigation consisted of children to MDCS-CC participants (N=1326). Subjects with missing data in any of the covariates used in analyses of associations between S1P and BP were excluded, resulting in 1046 eligible subject (Figure S1 in the Data Supplement). Ethical approval has been obtained for MOS at the Regional Ethics Committee in Lund (Dnr. 2012/594).

Clinical Assessment

Participants height (cm) and weight (kg) were measured in light indoor clothing. Resting BP (mm Hg) was measured after 10 minutes of rest in the supine position using an automatic device (Omron). A mean of 2 readings with 1 minute apart was calculated.

Smoking (yes/no), AHT, and alcohol usage were self-reported in a web-based questionnaire. Diabetes was defined as either self-reported diabetes diagnosis, use of antidiabetic medication, or fasting plasma glucose levels ≥7.0 mmol/L at 2 separate visits at the research facility.

Laboratory Assays

Blood samples were drawn after an overnight fast and analyzed for plasma glucose, creatinine, and cystatin C at the Department of Clinical Chemistry, Malmö, which is part of a national standardization and quality control system. Plasma glucose was measured using the HemoCue Glucose System (HemoCue, Ängelholm, Sweden). Plasma creatinine was measured using an enzymatic colorimetric assay with an IDMS-traceable calibrator on the Hitachi Modular P analysis system (Roche, Switzerland). Plasma levels of cystatin C were determined by an automated particle-based immunoassay using Hitachi Modular P analysis system and reagents from DAKO (Dako A/S, Denmark). Relative estimated glomerular filtration rate (eGFR) was calculated as a mean of eGFR derived from creatinine and eGFR derived from cystatin C and reported as mL/min per 1.73 m^2.

Proteomic Profiling

Proximity Extension Assay technique was applied to analyze plasma levels of proteins (OLINK Bioscience, Uppsala, Sweden) in a subsample of the population (N=444; consecutive subjects from March 6, 2013, until June 17, 2015, with complete data on all examinations). Four OLINK panels were analyzed: (1) Inflammation Panel https://www.olink.com/products/inflammation/ (2) Metabolism Panel https://www.olink.com/products/metabolism-panel/ (3) CVD II panel https://www.olink.com/products/cvd-ii-panel/ and (4) CVD III panel https://www.olink.com/products/cvd-iii-panel/ all comprising 92 proteins each within different domains (N=92×4=368). Proteins with ≥15% samples below limit of detection were excluded (N=20 for the metabolism panel; N=27 for the inflammation panel; N=7 for the CVDII panel; and N=4 for the CVDIII panel; Table S1). Additionally, 11 proteins were overlapping between panels; thus, only one of each marker was included in linear regression analyses (Table S2), resulting in 299 proteins that were taken forward to analyses. Validation data and coefficients of variance for all panels is available on the OLINK homepage (http://www.olink.com).

Animal Study

The investigations using research animals conform to the EU Directive 2010/63/EU for animal experiments and were conducted in accordance with European Animal Protection laws. All protocols were approved by the institutional ethical committee of Lund University (Dnr. 5.8.18/12637/2017). Commercially available male and female wild-type C57Bl/6N mice were obtained from Taconic (Ejby, Denmark) and housed under standard 12-hour:12-hour light-dark cycle with access to food and water ad libitum. Mice with a body weight ≥25 g were housed in groups of 4 to 5 mice per cage. Experimental groups were designed in a way to minimize stress for the animals and to guarantee maximal information using the lowest group size possible using a power calculation with type I error α=0.05 and power of 1-β >0.8 (80%) based on previous studies.[15] Hypertension was induced using Ang II (angiotensin II)–releasing osmotic pumps (Alzet-2006, AgnTho's, Sweden). In brief, animals were anesthetized with isoflurane (IsoFlo vet 100%, Sweden; 2.5% at 1.5 L/min in room air) for subcutaneous pump implantation containing Ang II (infusion rate 20 ng/kg per minute over 4 weeks). BP was measured biweekly in conscious mice using noninvasive tail-cuff plethysmography (CODA, EMKA, France), starting 1 week before pump implantation after a training period of 7 days. Weekly blood draws from the vena saphena were performed starting before pump implantation (=baseline) at 7 days intervals, and blood was collected in EDTA-coated tubes (Sarstedt, Germany). At termination, mice were anesthetized (isoflurane 2.5% at 1.5 L/min in room air) before euthanasia through cervical dislocation. Mesenteric arteries were dissected and immediately processed for RNA isolation using the Trizol method as per manufacturer's instructions and subsequent quantitative real-time polymerase chain reaction.

S1P Plasma Quantitation

S1P was extracted by mixing 10 µL of plasma with 90 µl of ice-cold methanol containing 22.2 nmol/L S1P-D7 (Avanti Polar Lipids / Merck, Darmstadt, Germany) as internal standard. After incubation on ice for 30 min precipitate was removed by centrifugation (20 000*g* for 10 minutes at 4 °C). The supernatant was analyzed by Liquid Chromatography Mass spectrometry on a 6495 QQQ instrument (Agilent Technologies, Sweden) essentially as described.[38] Extracts were separated on a 2.1×50 mm Acquity UPLC Peptide HSS T3 C18 column (Waters, Sweden) at a flow rate of 0.5 mL/min using eluents (A) water/0.1% formic acid/1 mmol/L ammonium formate, and (B)

methanol/0.1% formic acid/1 mmol/L ammonium formate, with a gradient of 20% A and 80% B to 100% B over 2 minutes, followed by 100% B for 6 minutes. By multiple reaction monitoring, MS/MS transitions of m/z 380 to 264 (with 380 to 82 as qualifier) for S1P, and 387 to 271 (with 387 to 82 as qualifier) for S1P-D7, were measured. A calibration curve consisting of 7 concentrations in the range of 0.1 to 2.4 μmol/L S1P in 4 % fatty acid-free BSA (bovine serum albumin) was generated in triplicates and measured during each session. Additionally, 3 plasma samples were used as quality control samples and measured during each session to ensure reproducibility.

Ex Vivo and In Vitro Testing

Endothelial cells of murine (bEND.3; ATCC CRL-2299) and human (HMEC-1; ATCC CRL-3243) origin were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin and MCDB131 supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% glutamine (2 mmol/L), 1% non-essential amino acids, 1% sodium pyruvate, 0.1% amphotericin B, and 0.1% human epidermal growth factor (10 ng/mL), respectively. Cell cultures were maintained at 37 °C with 5% CO_2 and split 1:4 at a seeding density of 10^6 cells. Cells were incubated with 1 μmol/L S1P or vehicle (4% fatty acid-free BSA) for 24 hours before processing for RNA isolation using the Trizol method as per manufacturer's instructions and subsequent quantitative real-time polymerase chain reaction. Mesenteric arteries isolated from male wild-type C57Bl/6N mice were cultured in DMEM (Gibco Life Technology, Sweden) containing 10% FBS (Gibco Life Technology, Sweden) and 1% penicillin-streptomycin (Sigma-Aldrich, Sweden) for 24 hours in the presence of 1 μmol/L S1P (Cayman Chemicals, BioNordika, Sweden) or vehicle (4% fatty acid-free BSA) before processing for RNA isolation and subsequent quantitative real-time polymerase chain reaction. Standard biochemical procedures were used for experiments involving reverse transcription polymerase chain reaction, quantitative polymerase chain reaction, and ELISA. Methodological details and primer sequences are provided in the Data Supplement.

Statistical Methods

Human Study

Comparisons between BP groups were performed using 1-way ANOVA for continuous variables and χ^2 tests for binary variables. For linear regression analyses of associations between S1P and BP, S1P was z-transformed. For associations between S1P and systolic BP and diastolic BP, linear regression analyses were performed unadjusted, adjusted for age and sex (Model 1), and further adjusted for body mass index (BMI), eGFR, diabetes status, AHT, smoking status, and alcohol usage (Model 2). Interaction analyses between age, eGFR, BMI, sex, and S1P were performed in linear regression analyses using a moderator variable. For associations between S1P and systolic BP ≥120 mm Hg; ≥130 mm Hg; and ≥140 mm Hg, logistic regressions were performed unadjusted, adjusted for age and sex (Model 1), and further adjusted for BMI, eGFR, diabetes status, AHT, smoking status, and alcohol usage (Model 2). For both linear and logistic regressions, $P<0.05$ was considered statistically significant.

For associations between proteins from the OLINK panels and S1P, NPX (log2) data were used, and S1P was log2-transformed accordingly as recommended for OLINK data (https://www.olink.com/content/uploads/2019/06/Olink-technical-comparisons-and-orthogonal-validation-v1.0.pdf). Linear regression analyses were performed unadjusted and adjusted for age and sex. To adjust for multiple testing, a Bonferroni-corrected P value of 0.05/299 ($P<1.67\times10^{-4}$) was applied. Pearson correlation analyses were performed with 2-tailed significance testing and computation of exact correlation coefficients (Pearson r). With correlation matrices being calculated for 4 different panels, multiple comparison adjustment was performed using Bonferroni-corrected P values for each correlation matrix individually ($P\leq7.81\times10^{-4}=0.05/64$ for the inflammation panel, $P\leq7.04\times10^{-4}=0.05/71$ for the metabolism panel, $P\leq5.95\times10^{-4}=0.05/84$ for the CVDII panel, and $P\leq5.75\times10^{-4}=0.05/87$ for the CVDIII panel). The panel-wise correction for multiple comparison avoids a (possible incorrect) rejection of significant correlations when correction is instead based on strict P values from the large amount of overall 299 markers. For methodological correctness, we always compare to the Bonferroni-corrected significance of all 299 markers used in the linear regression. Both Pearson correlation and linear regression analyses were performed using SPSS 26.0 (IBM).

Animal Study

All data are expressed as mean±SEM, where N is the number of animals. For assessment of differences in BP and plasma S1P levels over time, 2-way repeated measure ANOVA followed by Tukey post hoc testing was performed. For comparison of multiple independent groups, parametric 1-way ANOVA test was used, followed by Tukey post hoc test with exact P value computation. To compare two experimental groups in two sexes, 2-way ANOVA followed by Sidak's post hoc testing was performed. For comparison of 2 groups a 2-tailed unpaired t test was used. Differences were considered significant at error probabilities of $P\leq0.05$. All statistical analyses were performed using GraphPad software (Version 8.4.2).

Ex Vivo and In Vitro Studies

All data are expressed as mean±SEM, where N is the number of animals or independent in vitro experiments. For comparison of 2 groups, a 2-tailed unpaired t test was used. Differences were considered significant at error probabilities of $P\leq0.05$. All statistical analyses were carried out using GraphPad software (Version 8.4.2).

Visualization of Correlation Data

The matrix of pairwise correlations was used to construct a network graph with nodes given by the individual markers and edges drawn between nodes if their corresponding markers show a correlation of absolute value $r\geq0.3$. Edge width was weighted by correlation in absolute values. The network was grouped into clusters using Gephi 0.9.2,[39] which implements clustering by modularity taking edge weight into consideration.[40] Resolution was chosen around the default value of 1.0 so that a maximal modularity score is obtained. A separate t-distributed stochastic neighbor embedding (t-SNE)[41] analysis on the correlation data considers each variable as a data point with the correlation to all other variables as its features, defining a high-dimensional representation of the variables. We used the scikit-learn 0.23.1-implementation of t-SNE in Python (3.8.3) to learn a 2-dimensional representation suitable for visualization, which reflects the relation of similar correlations encoded as proximity in the high-dimensional data.

RESULTS

S1P Plasma Levels Associate With Systolic BP

S1P plasma levels were quantified in MOS-G2 participants with recorded systolic and diastolic BP information. Characteristics of the study population are presented in Table 1. There was a steady significant increase in S1P plasma levels as systolic BP values increased within cutoffs defined as <120 mm Hg; ≥120 mm Hg; ≥130 mm Hg, and ≥140 mm Hg (P=0.024; Table 1). Subjects with systolic BP ≥140 mm Hg were older and presented with significantly higher S1P plasma concentrations, higher BMI, higher diastolic BP, more frequent AHT and lower eGFR compared with subjects with BP <120 mm Hg. Furthermore, 1-way ANOVA analysis revealed no statistically significant differences in plasma levels of S1P between the sexes (P=0.163).

To explore associations between S1P and BP, linear regression analyses were performed revealing that each 1SD increment of S1P was associated with increasing systolic BP but not diastolic BP when adjusted for age, sex, BMI, eGFR, diabetes status, AHT, smoking, and alcohol usage (Table S3). The relationships between S1P and BP were not mediated by age (P=0.975), sex (P=0.495), eGFR (P=0.980), or BMI (P=0.834) as determined by interaction analyses. Furthermore, logistic regression analyses revealed that each 1SD increment of S1P was associated with systolic BP ≥120 mm Hg, ≥130 mm Hg, and ≥140 mm Hg in adjusted models, with similar odds ratios (Table 2).

To verify a possible positive correlation between plasma S1P and systolic BP, we longitudinally assessed plasma S1P concentrations in a murine model of slowly developing hypertension (induced by a low-pressor dose of 20 ng/kg per minute Ang II over the course of 4 weeks). In this model, BP steadily increased and established significance at 4 weeks after Ang II pump implantation (Figure 1A). Similar to systolic BP, plasma S1P concentrations were significantly elevated compared with baseline after 4 weeks of Ang II perfusion (Figure 1B). Thus, S1P plasma levels presented with a positive linear relationship to systolic BP in our murine model (r=0.7132, R^2=0.5086; P<0.0001; Figure 1C).

Proteomic Profiling Reveals Significant Associations Between S1P Plasma Levels and Inflammation, Metabolism, and CVD Markers

As hypertension is a major modifiable risk factor for CVD,[12,13,15,28] relates to metabolic disease,[25–27] and its pathophysiology strongly links to immune system activation and inflammation,[19–22] we tested if S1P plasma levels associate to biomarkers of CVD, metabolism, and inflammation in a subset of our study population (N=444). We categorized all markers with values above detection limit based on reported associations to cardiovascular or inflammatory diseases. Figure 2 illustrates an overview of biomarkers detected in each OLINK panel, incorporating all significant S1P correlations after single comparison (gray dots) and multiple

Table 1. Characteristics of the Study Population*

Variable	Total N=1046	<120 mm Hg N=537	120–129 mm Hg N=229	130–139 mm Hg N=153	≥140 mm Hg N=127	P value
S1P, nmol/L	770.7 (±173.7)	757.4 (±163.5)	771.6 (±172.8)	788.5 (±195.2)	804.1 (±184.9)	0.024
Age, y	51.7 (±7.9)	49.4 (±7.8)	52.7 (±7.5)	54.7 (±7.2)	56.6 (±6.2)	1.0×10^{-27}
Sex (women), N (%)	554 (53)	338 (62.9)	89 (38.9)	58 (37.9)	69 (54.3)	1.3×10^{-11}
BMI, kg/m^2	26.6 (±4.5)	25.6 (±4.2)	27.3 (±4.7)	27.8 (±4.6)	28.2 (±4.3)	4.3×10^{-13}
SBP, mm Hg	121 (±16)	109 (±7)	124 (±3)	134 (±3)	152 (±10)	…
DBP, mm Hg	76 (±10)	70 (±6)	77 (±6)	83 (±6)	90 (±8)	1.3×10^{-195}
eGFR, mL/min per 1.73 m2	81.4 (±9.7)	82.9 (±9)	82 (±10)	79 (±10)	76 (±11)	8.0×10^{-11}
Diabetes status (yes), N (%)	43 (4.1)	11 (2.0)	10 (4.4)	15 (9.8)	7 (5.5)	2.7×10^{-4}
AHT (yes), N (%)	137 (13.1)	28 (5.2)	37 (16.2)	40 (26.1)	32 (25.2)	3.5×10^{-15}
Smoking (yes), N (%)	94 (9.0)	42 (7.8)	20 (8.7)	17 (11.1)	15 (11.8)	0.393
Alcohol usage, N (%)						0.016
Never	69 (6.6)	41 (7.6)	8 (3.5)	11 (7.2)	9 (7.1)	
Monthly or less	169 (16.2)	104 (19.4)	30 (13.1)	14 (9.2)	21 (16.5)	
2–4 times a month	377 (36.0)	192 (35.8)	84 (36.7)	63 (41.2)	38 (29.9)	
2–3 times a week	368 (35.2)	169 (31.5)	93 (40.6)	52 (34.0)	54 (42.5)	
≥4 times a week	63 (6.0)	31 (5.8)	14 (6.1)	13 (8.5)	5 (3.9)	

Statistical significance is given by exact P value computation after multiple comparisons between different blood pressure groups. AHT indicates antihypertensive treatment; BMI, body mass index; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; S1P, sphingosine-1-phosphate; and SBP, systolic blood pressure.

*Values are means (±SD) or n (%).

Table 2. S1P and Systolic Blood Pressure Associations*

	≥120 mm Hg		≥130 mm Hg		≥140 mm Hg	
Model	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Unadjusted						
S1P	1.17 (1.04–1.32)	0.011	1.21 (1.06–1.38)	0.005	1.23 (1.03–1.46)	0.021
Model 1						
S1P	1.14 (1.01–1.30)	0.047	1.19 (1.03–1.37)	0.016	1.21 (1.01–1.46)	0.037
Age	1.10 (1.08–1.12)	1.0×10^{-21}	1.10 (1.08–1.13)	1.7×10^{-19}	1.11 (1.08–1.15)	5.4×10^{-13}
Sex	0.41 (0.31–0.53)	1.9×10^{-11}	0.66 (0.49–0.88)	0.004	1.12 (0.76–1.65)	0.563
Model 2						
S1P	1.15 (1.01–1.32)	0.046	1.21 (1.04–1.40)	0.012	1.23 (1.02–1.48)	0.034
Age	1.09 (1.06–1.11)	9.0×10^{-14}	1.08 (1.06–1.11)	6.7×10^{-11}	1.10 (1.06–1.13)	6.2×10^{-8}
Sex	0.48 (0.36–0.64)	2.6×10^{-7}	0.77 (0.57–1.05)	0.098	1.20 (0.80–1.81)	0.387
BMI	1.09 (1.06–1.13)	1.0×10^{-7}	1.07 (1.04–1.11)	6.3×10^{-6}	1.07 (1.03–1.12)	0.002
eGFR	1.01 (0.99–1.02)	0.643	0.99 (0.97–1.01)	0.202	0.98 (0.96–1.00)	0.048
Diabetes status	1.00 (0.46–2.20)	0.993	0.86 (0.43–1.72)	0.676	1.71 (0.69–4.23)	0.249
AHT	2.89 (1.80–4.63)	1.1×10^{-5}	2.12 (1.41–3.20)	3.1×10^{-4}	1.47 (0.89–2.43)	0.130
Smoking	1.25 (0.77–2.02)	0.377	1.27 (0.77–2.11)	0.348	1.14 (0.60–2.16)	0.686
Alcohol						
Never	1.09 (0.50–2.40)	0.828	1.48 (0.63–3.44)	0.366	2.11 (0.62–7.17)	0.232
Monthly or less	0.98 (0.51–1.91)	0.961	0.94 (0.45–1.97)	0.877	2.24 (0.75–6.72)	0.149
2–4 times a month	1.43 (0.79–1.91)	0.238	1.30 (0.68–2.47)	0.433	1.74 (0.63–4.83)	0.286
2–3 times a week	1.53 (0.84–2.76)	0.164	1.27 (0.67–2.41)	0.466	2.54 (0.94–6.92)	0.067
≥ 4 times a week	0.92 (0.42–2.02)	0.828	0.79 (0.41–1.50)	0.466	0.57 (0.21–1.59)	0.286

AHT indicates antihypertensive treatment; BMI, body mass index; eGFR, estimated glomerular filtration rate; OR, odds ratio; and S1P, sphingosine-1-phosphate.

*Values are OR and 95% CI and significance values (*P*) for logistic regression analyses.

comparison testing (black dots). Pearson correlation analysis revealed 23 significant associations between plasma S1P and inflammation panel markers (panel-specific Bonferroni correction), 21 of which were validated with Bonferroni correction for testing all 299 markers (Figure 2A and Figure S2A). Altogether 23 metabolism panel markers significantly correlated with plasma S1P (significant after both for panel-specific Bonferroni correction and for testing all 299 markers Figure 2B and Figure S2B). CVDII and CVDIII panels presented with 22 and 24 proteins that correlated significantly with S1P levels (Bonferroni-corrected for individual panels), of which 21 and 16 remained significant after Bonferroni correction for testing all 299 markers (Figure 2C/2D and Figure S2C/S2D). Overall, Pearson correlations revealed a total of 185 correlations with S1P levels across all panels, of which 92 and 81 remained significant after Bonferroni correction for the number of panel-specific tests and all 299 tests, respectively (Figure S2). Correction for age and sex in the linear regression analyses resulted in the same number of significant associations for the individual biomarker panels. A full list of all unadjusted and age- and sex-adjusted associations in linear regression analyses between S1P and 299 proteins from all 4 panels is presented in Table S4.

When applying a cutoff defined as $r \geq 0.3$ or $r \leq -0.3$ to determine the strongest significant Pearson correlations[42,43] with plasma S1P in all 4 panels, a total of 29 markers were extracted that all revealed significant associations with S1P in linear regression analyses. Sixteen out of 29 strongly correlated markers belong to CVDII and CVDIII panels, 9 to the metabolism panel, and 4 to the inflammation panel. Figure 3 illustrates individual correlation networks of all 4 OLINK panels. Modularity clustering was applied to all networks as an algorithmic approach to detect communities of markers with strong pairwise correlations within a community and less frequent intercorrelations to markers of other communities. For the inflammation panel, marker clusters can be linked to T-cell homeostasis, immune cell homeostasis and chemotaxis (cluster A), neutrophil chemotaxis and angiogenesis (cluster B), or regulation of immune responses and association to CVD (cluster C), respectively (Figure 3A). S1P associations belong to cluster B and link to T-cell metabolism and apoptosis. Similarly, modularity clustering of metabolism panel markers detected 3 groups, separating markers associated to cell adhesion (cluster A), apoptosis and cellular stress response (cluster B), or cellular metabolism (cluster C). S1P associations link to markers representing cluster B (Figure 3B). CVDII panel clusters represent

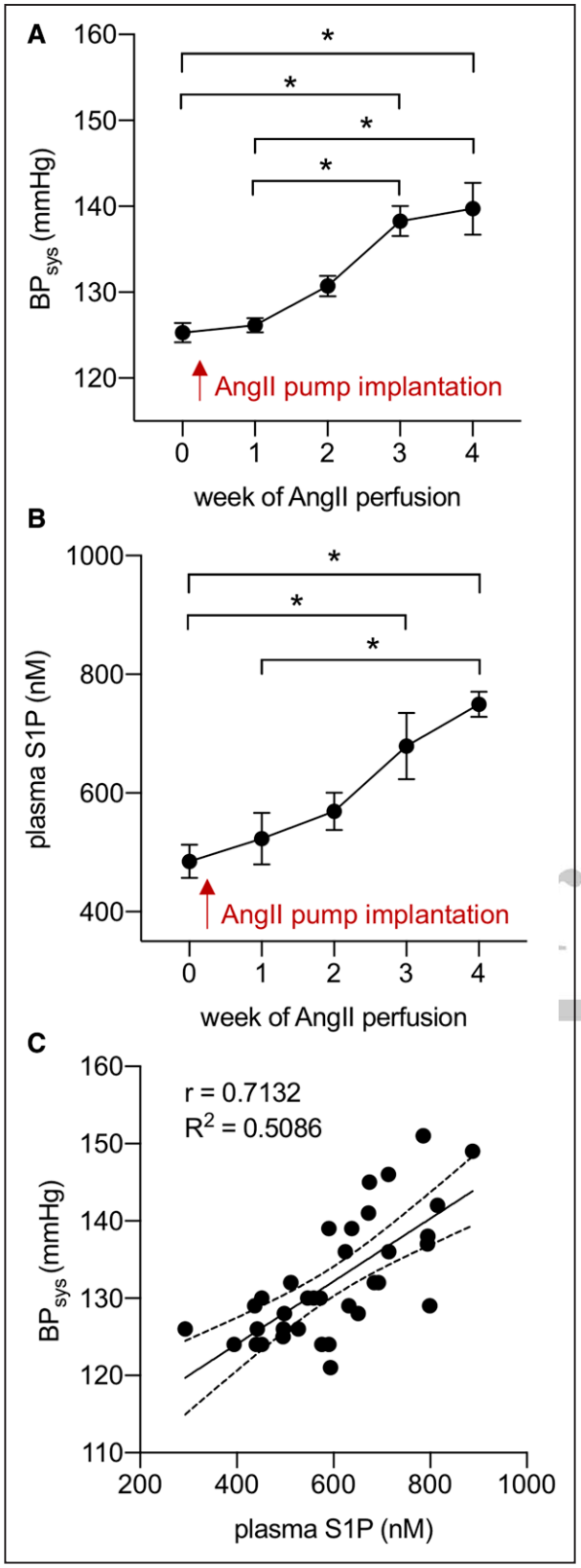

Figure 1. S1P (Sphingosine-1-phosphate) plasma concentrations increase with systolic blood pressure (BP) in a mouse model of Ang II (angiotensin II)–induced hypertension.
A, Longitudinal assessment of systolic BP (BP$_{sys}$ in mm Hg) in responses to a low-pressor dose of Ang II (*Continued*)

features linking to inflammation and metabolism associated to atherosclerosis, plaque development and cardiovascular events (cluster A), angiogenesis (cluster B), vascular dysfunction and inflammation (cluster C), the latter including all S1P associations of this panel (Figure 3C). For the CVDIII panel, marker clusters link to vascular dysfunction, remodeling and inflammation (cluster A), endothelial activation and inflammation (cluster B) or development of atherosclerosis and cardiovascular events (cluster C), respectively (Figure 3D). Strongest S1P associations belong to cluster B.

Modularity clustering applied to a network constructed from correlation factors of markers of all panels combined, separates markers into 5 distinct clusters, which can be linked to different processes involved in inflammation and CVD previously reported by clinical and preclinical studies (Figure 4). All significant S1P correlations are highlighted in red and associate to clusters 2, 3, and 4, representing markers linked to vascular inflammation, cell adhesion, immune cell metabolism, and chemotaxis as well as atherosclerosis and cardiovascular events. To support the significance and stability of the found clusters, an application of a t-SNE visualizes the high-dimensional correlation data in 2 dimensions, revealing an underlying data structure that can be compared with the clusters (Figure S3). While the positioning of markers within a modularity cluster is arbitrary, the t-SNE locations of markers are meaningful where proximity is explained by similarity in correlations with other markers. The t-SNE arranges the markers into 9 visual groups that were linked to different processes involved in inflammation and CVD. When comparing t-SNE groups with the modularity clusters, 81%–92% marker overlap was observed between 5 t-SNE groups (groups 1–3, 7, and 9; Figure S3) and clusters 1 to 5 (Figure S4). All S1P correlations are associated to groups 7 and 8, resembling marker groups linked to vascular inflammation, immune cell homeostasis, and cell adhesion or endothelial inflammation and thrombosis, respectively. Marker overlap with cluster 3 (92%), where most markers can be linked to vascular inflammation, immune cell homeostasis, and cell adhesion, largely confirms the meaningfulness of the modularity clusters. Correlation data used for all visualizations are presented as correlation matrices (Figure S4).

Figure 1 Continued. (20 ng/kg per minute) in wild-type (WT) mice over the course of 4 wk. **B**, Longitudinal plasma S1P quantification in WT mice developing hypertension over the course of 4 wk. **C**, Linear regression analysis of BP$_{sys}$ (mm Hg) and plasma S1P concentrations of WT mice developing hypertension in response to a low-pressor dose of Ang II (20 ng/kg per minute). In **A** and **B**, N=7; *$P \leq 0.05$ compared with baseline after 2-way repeated measure ANOVA followed by Dunnett post hoc testing. In **C**, N=7 per group and time point; linear regression analysis of association between plasma S1P and BP$_{sys}$ levels (mm Hg) with a calculated goodness of fit measurement (R^2), Pearson *r*, and exact *P* value computation ($P<0.0001$).

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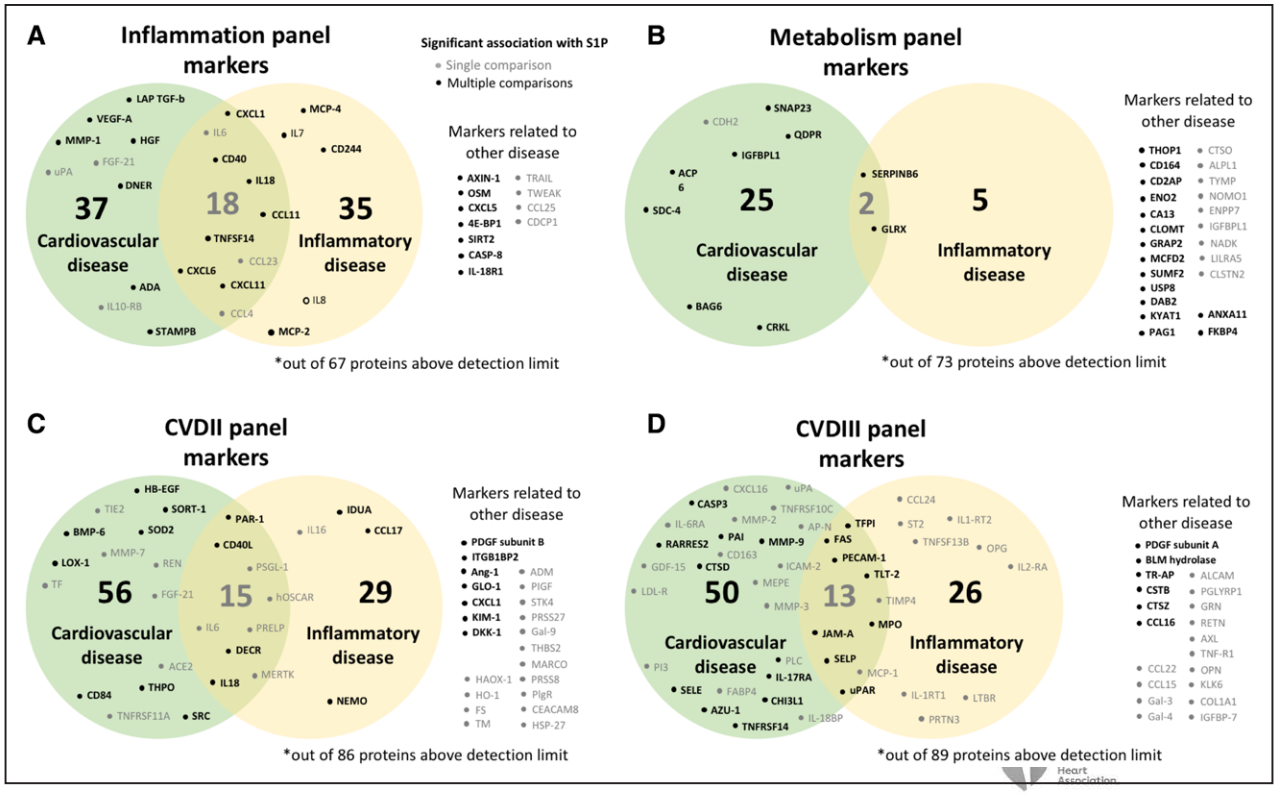

Figure 2. S1P (Sphingosine-1-phosphate) significantly associates with various biomarkers in inflammation, metabolism, and cardiovascular disease (CVD) OLINK panels.

Overview of significant S1P correlations and associations among 299 proteins from **A** inflammation, **B** metabolism, **C** CDVII, and **D** CVDIII OLINK panels. The numbers represent all panel markers with previously reported links to cardiovascular (green) or inflammatory disease (yellow), including overlapping markers in each panel. Significant S1P associations to these markers after Pearson correlation and linear regression analyses are presented for 2-tailed test (gray dots), and for multiple comparisons with Bonferroni correction of *P* values (black dots). N=444.

To experimentally test the effect of S1P on marker expression profiles, we performed in vitro and ex vivo experiments utilizing endothelial cells of murine and human origin that were treated with 1 μmol/L S1P. As illustrated in Figure 4B through 4D, exposure to S1P significantly increased the expression of markers characteristic for angiogenesis and endothelial activation such as PAI (plasminogen activation inhibitor 1; Figure 4B), PECAM-1 (platelet endothelial cell adhesion molecule 1; Figure 4C), and SELP (P-selectin; Figure 4D) in cultured human or mouse endothelial cells. All markers presented strongest positive associations with plasma S1P in our study cohort (r=0.409, β=0.409 for PAI-1; r=0.389, β=0.378 for PECAM-1, and r=0.383, β=0.384 for SELP). Remarkably, mesenteric arteries isolated from hypertensive mice presented with augmented PECAM-1 and SELP mRNA expression levels when compared with normotensive controls (Figure 4E and 4F), strongly suggesting a link between plasma S1P and hypertension-associated vascular dysfunction and inflammation as systolic BP significantly correlated with plasma S1P levels in this model (Figure 1C). Although cross-sectional associations do not allow conclusions on causality, several different markers increased expression after exposure to high S1P concentrations, including small resistance artery Ang-1 (angiopoietin 1), endothelial cell Casp3 (caspase 3), endothelial cell IL (interleukin)-18, and monocytic cell CD40 (Figure S5).

Proteomic Profiling Discloses Sex-Specific S1P Associations for a Subset of Markers

As sex-dependent differences have been discussed for various S1P responses,[44] we investigated the possibility of sex-specific S1P associations of all protein markers tested in our study. In single comparisons, we identified 66 significant sex-dependent S1P correlations, out of which 32 showed stronger associations with male sex and 34 with female sex (Table S5). After panel-specific correction for multiple comparisons, the majority of sex-specific differences were dictated by female sex (3 of 4 in the inflammation panel, 3 of 3 in the CVDII panel, and 1 of 2 in the metabolism panel), while only one male-specific association remained significant after correction for multiple comparisons in the CVDIII panel. Eight out of 10 sex-specific associations were confirmed with strict Bonferroni correction

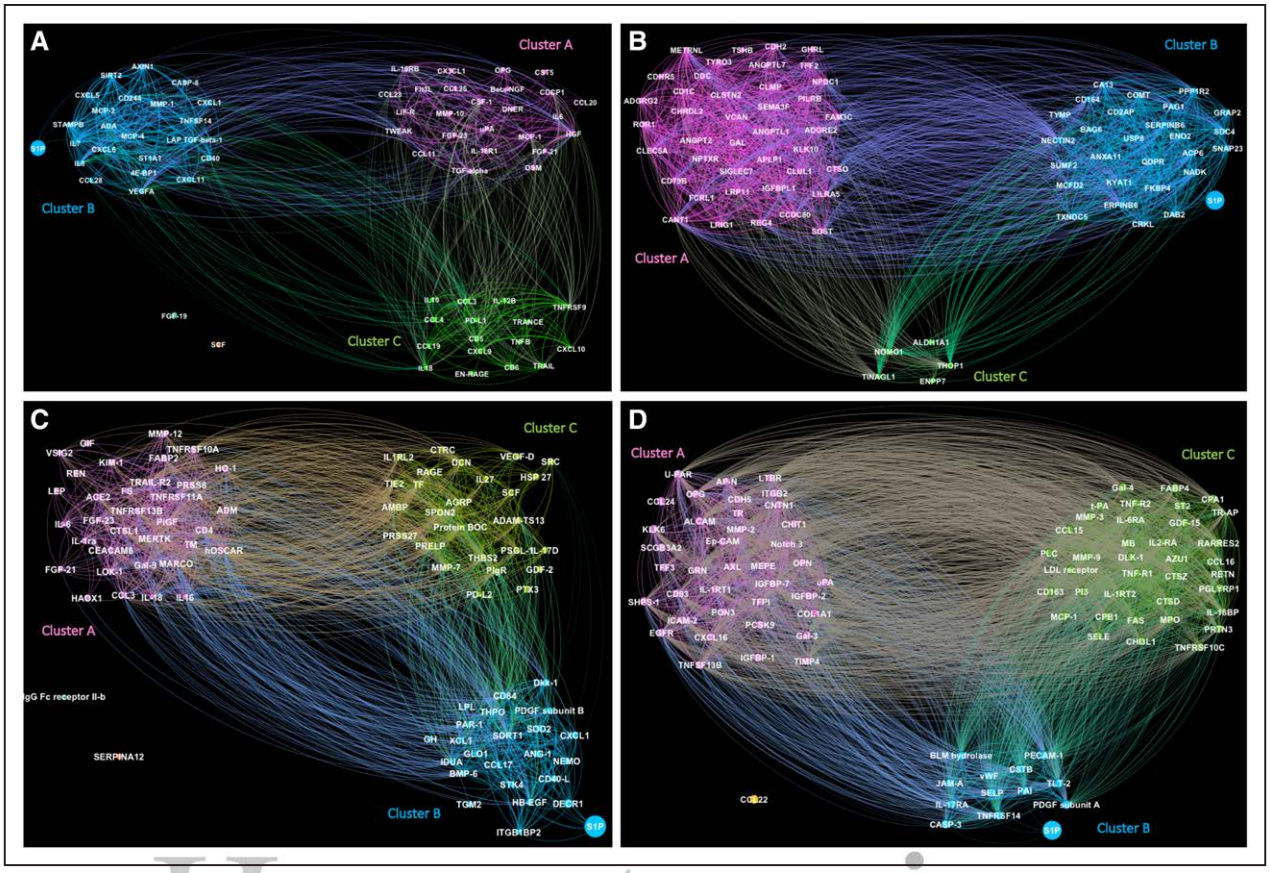

Figure 3. Correlation networks showing significant S1P (sphingosine-1-phosphate) correlations with biomarkers in the inflammation, metabolism, and cardiovascular disease (CVD) OLINK panels.
Modularity clustering applied to correlation networks for **A** inflammation panel (modularity =0.271), **B** metabolism panel (modularity =0.273), **C** CVDII panel (modularity =0.189), and **D** CVDIII panel (modularity =0.03) where each node represents a marker and edges between nodes a correlation factor of $r \geq 0.3$ or $r \leq -0.3$. Coloring according to cluster and edge width is weighted by correlation.

for all 299 markers (Figure 5A). Among them IL-18, which exerts apparent vascular and immune responses during hypertension and associates to adverse cardiovascular events, presented with a significantly stronger correlation in females (r=0.278 and β=0.538; P=1.80×10^{-5}) compared with males (r=0.157 and β=0.283; P=0.021). Investigating the relation between IL-18 and S1P in a controlled experimental setting revealed a similar sex-specific difference as evident by higher IL-18 plasma levels in female hypertensive mice compared with their male counterparts (Figure 5B). Testing a linear relationship between IL-18 and plasma S1P disclosed an extremely strong association in female mice (r=0.9260, R^2=0.8574; P<0.0001) but not in male mice (r=0.2175, R^2=0.0473; P=0.436) in our model (Figure 5C). Similar to observations in our human cohort, plasma S1P responses to BP increases did not differ sex specifically (Figure 5D). In neither mice nor humans did IL-18 associate to BP in either sex (male mice: r=0.257, R^2=0.066, P=0.354; female mice r=−0.222, R^2=0.491, P=0.427; men: r=0.070, P=0.308 and β=1.27; P=0.308; women: r=0.076, P=0.257 and β=2.90, P=0.257).

DISCUSSION

For the first time, we show a significant association between plasma S1P and systolic BP levels in a large human cohort study (N=1046) and validate these findings in a longitudinally conducted preclinical murine hypertension study. Additionally, our data provide the first evidence of significant associations between plasma S1P and multiple cardiovascular, inflammation, and metabolism biomarkers assessed by proteomic profiling of 444 MOS participants. Some of these markers present with marked sex-specific interactions. Taken together, our translational findings strongly suggest a link between S1P plasma concentrations and systolic BP and encourage further prospective studies that investigate S1P's potential as therapeutic target or risk marker in hypertensive disease.

The bioactive phosphosphingolipid S1P plays a critical role in both the vascular and the immune system and has proven involvement in experimental hypertension.[12,15,45,46] Specifically, one of the enzyme generating S1P, SphK2 (sphingosine kinase 2), presents as a key player in mediating plasma S1P responses to Ang II, immune cell

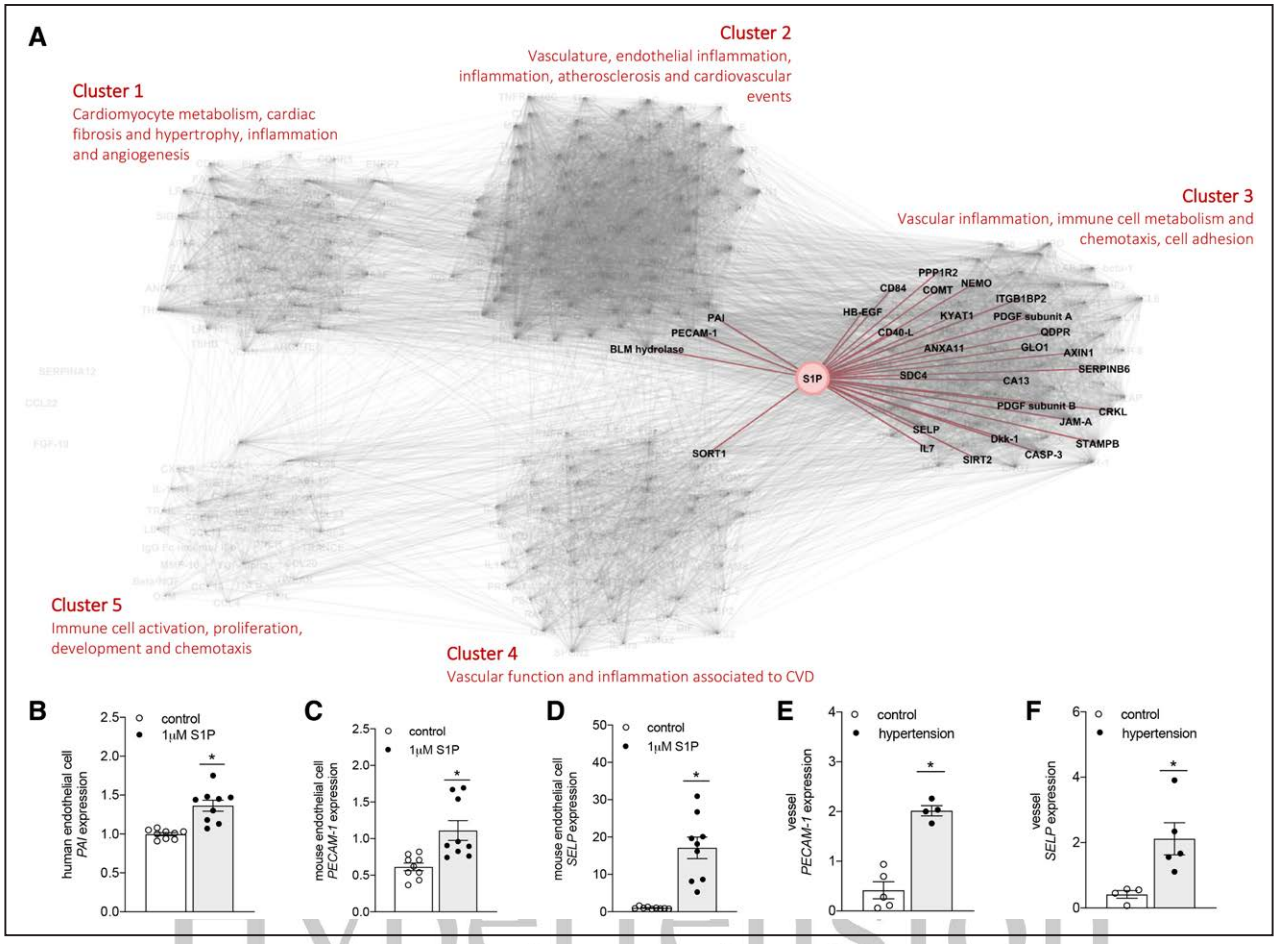

Figure 4. S1P (Sphingosine-1-phosphate) increases various markers of inflammation and vascular dysfunction.
A, Modularity clustering applied to a network constructed from correlation factors of markers ($r \geq 0.3$ or $r \leq -0.3$) from inflammation, metabolism, cardiovascular disease (CVD)II, and CVDIII OLINK panels combined. Significant S1P correlations highlighted in red. Clusters were categorized based on previously reported marker involvement in inflammation and CVD. **B**, Augmentation of endothelial activation marker mRNA expression in human endothelial cells in response to 1 µmol/L S1P (6 h). **C** and **D**, Augmentation of endothelial activation marker mRNA expression in murine endothelial cells in response to 1 µmol/L S1P (12 h). **E** and **F**, Augmentation of endothelial activation marker mRNA expression in mesenteric arteries isolated from normotensive and hypertensive mice. In **A**, N=444. In **B**–**D**, N=3 per group in triplicates; *$P \leq 0.05$ after single unpaired comparisons. In **E** and **F**, N=5 per group; *$P \leq 0.05$ after single unpaired comparisons.

mobilization, and vascular dysfunction, and hence, contributes to the development of overt hypertension.[15] Thus far, human-based studies investigating S1P associations with BP were lacking. Our study is the first to reveal associations between increasing S1P plasma levels and systolic BP cutoffs of <120 mm Hg, ≥120 mm Hg, ≥130 mm Hg, and ≥140 mm Hg. Concurrently, the effect size of S1P association with systolic BP in humans is comparable to those of well-established hypertension-associated biomarkers such as renin, ST2 (soluble interleukin 1 receptor-like 1), and IL-6. Together, these promising results call for further prospective studies to investigate S1P's potential suitability as predictive marker for identifying subjects at high risk of incident hypertension in the population. As the first study showing significant associations between plasma S1P and systolic BP in a large population cohort, it is reassuring that the mean S1P plasma levels measured in our human study population validate those recently published for a study group comprising 174 healthy participants with a median age of 45.5 years.[47] Similar to our study, neither S1P plasma nor serum levels were affected by sex, age, or BMI.[47,48]

Previously, S1P was assigned with marker capacity for coronary artery disease.[49] The authors speculated whether S1P might be a marker for inflammatory processes associated with coronary disease or an ischemic marker. Here, we provide the first evidence of significant associations between plasma S1P and multiple CVD, inflammation, and metabolism markers in a relatively young study population with very few cardiovascular incidents, suggesting that S1P might associate to pathogenesis (eg, inflammatory processes) rather than end points. In accordance, modularity clustering arranged the strongest S1P associations into marker communities linked to vascular inflammation, immune cell homeostasis, cell adhesion, endothelial inflammation, and thrombosis. PECAM-1 as multifunctional vascular cell adhesion molecule,[50] presented with positive associations to

A

Inflammation panel	Men (N=216)			Women (N=228)		
	Pearson's r	β	P	Pearson's r	β	P
MMP-1	0.258	0.911	$1.28x10^{-4}$	0.202	0.663	0.002
CD40	0.200	0.345	0.003	0.254	0.382	$1.02x10^{-4}$
TWEAK	0.039	0.050	0.571	0.246	0.283	$1.78x10^{-4}$
CD244	0.160	0.235	0.019	0.245	0.308	$1.84x10^{-4}$
Metabolism panel	**Pearson's r**	**β**	**P**	**Pearson's r**	**β**	**P**
ENO2	0.250	0.601	$1.97x10^{-4}$	0.316	0.677	$7.97x10^{-7}$
SUMF2	0.284	0.654	$2.10x10^{-5}$	0.196	0.435	0.003
CVDII panel	**Pearson's r**	**β**	**P**	**Pearson's r**	**β**	**P**
LOX-1	0.194	0.305	0.004	0.250	0.423	$1.21x10^{-4}$
IL18	0.157	0.283	0.021	0.278	0.538	$1.80x10^{-5}$
GLO1	0.241	0.619	$3.41x10^{-4}$	0.389	0.929	$8.93x10^{-10}$
CVDIII panel	**Pearson's r**	**β**	**P**	**Pearson's r**	**β**	**P**
CTSD	0.276	0.567	$4.0x10^{-5}$	0.189	0.271	0.009

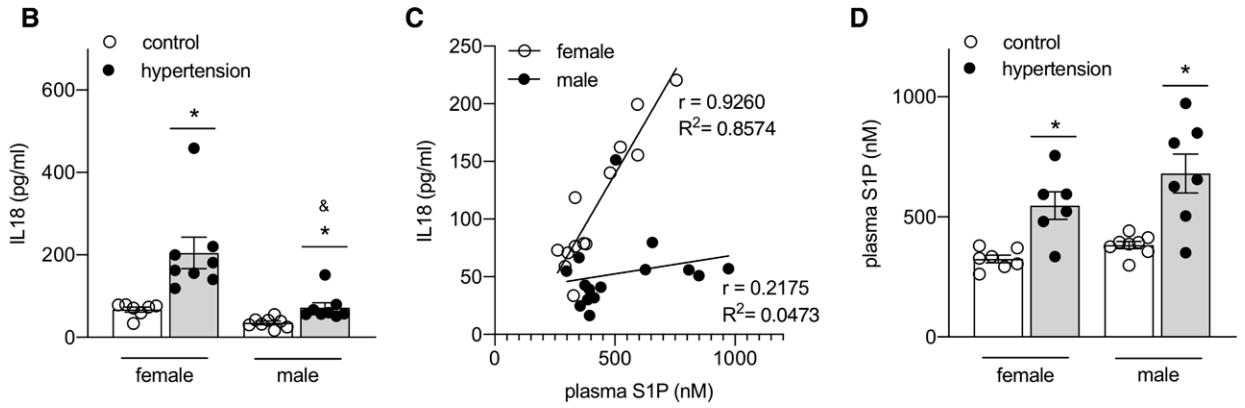

Figure 5. Plasma S1P (sphingosine-1-phosphate)-IL (interleukin)-18 associations differ sex dependently.
A, Biomarkers with sex-specific associations with plasma S1P. The association-determining sex highlighted in orange. **B**, Plasma IL-18 concentrations in normotensive and hypertensive female and male wild-type (WT) mice. **C**, Linear regression showing associations between plasma S1P and IL-18 in normotensive and hypertensive female and male WT mice. **D**, Plasma S1P concentrations in normotensive and hypertensive female and male WT mice. In **A**, N=444 (N=216 for men and N=228 for women). Multiple comparison adjustment was performed using Bonferroni-corrected *P* values of 0.05/299. For the inflammation panel, 2 additional significant sex-specific association with plasma S1P are found significant according to panel-specific Bonferroni correction of 0.05/64 ($P \leq 7.81 \times 10^{-4}$) that are highlighted in yellow. In **B** and **D**, N=7–8 per group; *$P \leq 0.05$ compared with same sex control and &$P \leq 0.05$ compared with female hypertension group after 2-way ANOVA followed by Sidak's post hoc testing. In **C**, N=6–8 per group; Pearson correlation with goodness of fit and r computation; significance from zero was calculated for female ($P<0.0001$) but not male mice ($P=0.4361$).

S1P in humans. A t-SNE visualization, where locations of markers are meaningful as proximity is explained by similarity in correlations with other markers, grouped PECAM-1 in close proximity to other makers related to endothelial inflammation, thrombosis, atherosclerosis, and cardiovascular events, confirming S1P-PECAM-1 involvement in endothelial damage and deregulation of inflammatory responses at the blood-endothelial interface.[51] The observed augmentation of PECAM-1 expression in response to excess exogenous S1P in vitro, in vivo, and ex vivo supports previous studies, demonstrating PECAM-1 as a downstream target for S1P signaling in human endothelial cells.[52] Together with our findings that show elevated resistance artery PECAM-1

expression and increased plasma S1P in hypertensive mice, these data are supportive of potential contributions of S1P-PECAM-1 deregulations in hypertensive disease. Similarly, SELP that plays an essential role in the recruitment of leukocytes during inflammation[53] presented with strong S1P associations in our human cohort and was upregulated in human and murine endothelial cells following exogenous S1P treatment. In our visualization approaches SELP clusters together with markers of vascular inflammation and cell adhesion and in close proximity to PECAM-1, respectively. This is particularly intriguing since soluble SELP levels are elevated among healthy women at risk for future vascular events[54] thus, encouraging the consideration of S1P as therapeutic target or risk marker for hypertensive disease.

A potential sex-specificity for S1P plasma concentrations is controversially discussed with equally many studies suggesting sex-dependent differences as those reporting similar S1P plasma levels between the sexes.[44,47,48,55–58] A study investigating S1P levels, sex, and pre/postmenopausal status in 108 healthy participants demonstrated a significant association between plasma S1P and estrogen, with higher S1P plasma concentration in premenopausal than postmenopausal women.[44] Evaluating a much smaller study population, the authors assessed plasma S1P levels in individuals in an age range between 16 and 55 years as compared to our study population with a narrow age range, 51.7 (±7.9) years, and presumably a predominance of postmenopausal women. Nonetheless, the herein observed sex independence of S1P concentrations, specifically in respect to disease, requires verification in even larger cohorts. In light of known sex-specific differences in BP control,[59] experimental studies highlighted the critical role of inflammation (ie, regulatory T cells and T helper 17 cells) in mediating such differences.[60] Despite the relative sex independence of plasma S1P in our study, some of the herein identified S1P associations with proteomic markers presented with sex-specific interactions. Among them, IL-18, which has been suggested as independent predictor of cardiovascular events in subjects with metabolic syndrome[61] and chronic kidney disease,[62] is involved in destabilization of atherosclerotic plaques[63] and was linked to essential hypertension.[64] The herein observed sex-specific differences in S1P-IL-18 associations are interesting as they are independent of BP (ie, correlation and linear regression analyses revealed no significant associations between IL-18 and BP in either sex). In respect to BP, only one experimental study thus far reported significantly higher IL-18 mRNA expression in the renal cortex of female spontaneously hypertensive rats compared with age-matched males[65] despite 10 to 15 mm Hg higher BP in male compared with female rats.[66] This is particularly interesting since BP in men significantly differs from women in our study cohort (125 mm Hg (±14) and 119 (±17) for men and women respectively; $P=7.5\times10^{-10}$). Similar female-driven increases in the renal cortex of spontaneously hypertensive rats were observed for CD40 expression,[65] which positively associates with S1P plasma levels in women but not men in our study. Thus, our findings show that BP-independent sex-specific S1P associations to different inflammation, metabolism, and CVD markers exist, suggestive of sex-specific differences in immune and vascular responses during hypertension or its development with potential significance for cardiovascular health that might only be unveiled when directly comparing to S1P plasma levels.

Strengths and Limitations

By studying a general population and adjusting for risk factors for hypertension, we demonstrated that S1P plasma level are associated with increased systolic BP in humans. Furthermore, we substantiated these findings in an animal model of hypertension. However, since BP regulation is multifactorial, drawing conclusions about associations should be done with caution. The present study shares limitations common to all cross-sectional studies as no conclusion of causality can be drawn. Moreover, the MOS study was performed in subjects of mainly Swedish descent, and the conclusions may not be generalizable to all populations or ethnicities. Also, since the MOS study is ongoing and participants still fairly young, we were unable to investigate possible association of S1P with incident disease (eg, incident hypertension or CVD).

Proteomic profiling of 4 different OLINK panels (inflammation, metabolism, CVDII, and CVDIII) was performed using interplate controls for markers within the individual panels, but no standardization was performed to compare between the different panels. By analyzing markers that appeared in more than one panel, a high degree of similarity (ie, correlation of the same marker between 2 panels) was observed for inflammation, metabolism, and CVDII panels ($r=0.8$). In a few cases of the CVDIII panel, however, lower correlation coefficients were observed ($r=0.45$ for 2 extreme cases). We identified a technical variation associated to the OLINK data as reason for the low correlation between duplicate markers, which has been reported before[67] and which became substantial for some markers with a biological variance below 0.4 NPX. Thus, correlations to markers outside the panels, in particular S1P, should not be expected to markedly surpass correlation factors of duplicate markers, and some associations may remain undetected due to the commonly used cutoff $r>0.3$. Additionally, we observed a small bias on correlation factors within a panel. This induces a higher chance for markers within one panel to fall into the same cluster when combining all 4 panels. To mitigate the role of the technical error in the cluster formations, we performed additional individual panel clustering for data interpretation.

Perspective

For the first time, we show a clear relationship between increments in plasma S1P and increasing systolic BP in a large human population cohort. Together with the observed associations between plasma S1P and multiple CVD, inflammation, and metabolism biomarkers, this is suggestive of S1P's biomarker potential. Our findings strongly encourage further prospective studies to investigate S1P's capacity as predictive marker for hypertensive disease.

ARTICLE INFORMATION

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Disclosures

None.

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