Proteomic Profiling for Cardiovascular Biomarker Discovery in Orthostatic Hypotension

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Abstract—Orthostatic hypotension (OH) has been linked with higher incidence of cardiovascular disease, but little is known about the mechanisms behind this association. We aimed to identify cardiovascular disease biomarkers associated with OH through a proteomic profiling approach. Seven hundred seventy-eight patients with unexplained syncope or orthostatic intolerance underwent head-up tilt test and supine blood samples. Of these, 220 met diagnostic criteria of OH, and 179 demonstrated normal hemodynamic response during head-up tilt test. Blood samples were analyzed by antibody-based Proximity Extension Assay technique simultaneously measuring 92 cardiovascular disease-related human protein biomarkers. The discovery algorithm was a sequential 2-step process of biomarker signature identification by supervised, multivariate, principal component analysis and verification by univariate ANOVA with Bonferroni correction. Patients with OH were older (67 versus 60 years; $P<0.001$) and more likely to be women (48% versus 41%; $P>0.001$) but did not differ from OH-negative patients in medical history. Principal component analysis identified MMP-7 (matrix metalloproteinase-7), TM (thrombomodulin), MB (myoglobin), TIM-1 (T-cell immunoglobulin and mucin domain-1), CASP-8 (caspase-8), CXCL-1 (C-X-C motif chemokine-1), Dkk-1 (dickkopf-related protein-1), lectin-like LOX-1 (oxidized low-density lipoprotein receptor-1), PlGF (placenta growth factor), PAR-1 (proteinase-activated receptor-1), and MCP-1 (monocyte chemotactic protein-1) as the most robust proteomic signature for OH. From this proteomic feature selection, MMP-7 and TIM-1 met Bonferroni-adjusted significance criteria in univariate and multivariate regression analyses. Proteomic profiling in OH reveals a biomarker signature of atherothrombosis and inflammation. Circulating levels of MMP-7 and TIM-1 are independently associated with OH and may be involved in cardiovascular disease promotion. **(*Hypertension*. 2018;71:00-00. DOI: 10.1161/HYPERTENSIONAHA.117.10365.)** ● **Online Data Supplement**

Key Words: biomarkers ■ cardiovascular diseases ■ hypotension, orthostatic ■ proteomics ■ thrombosis

Orthostatic hypotension (OH) is the main symptom of cardiovascular autonomic dysfunction and has an incidence of ≈6% in the general population.[1,2] The diagnosis of OH is frequently found among older patients hospitalized because of syncope, falls, and fractures, which infers large costs for society, whereas the cause of OH is commonly not clear with no specific treatment.[3]

The prevalence of OH increases with advancing age and comorbidities, including hypertension, diabetes mellitus, and renal failure.[2] In population-based studies, OH has been linked with higher rates of myocardial infarction,[4,5] stroke,[6] and heart failure,[7] as well as with increased mortality.[8] Recent reports suggest that the presence of OH may be associated with left ventricular hypertrophy,[9] hypercoagulability,[10] and neuroendocrine hyperactivation–increased levels of endothelin and vasopressin.[11] However, despite these identified relationships, the mechanisms linking OH and autonomic dysfunction with increased risk of cardiovascular disease (CVD) are not fully understood.

In recent years, new technologies yielding broad protein biomarker screening have been introduced offering the hope to understand better the pathophysiological mechanisms of CVD.[12,13] A novel targeted proteomics approach using the proximity extension technique revealed several new associations of related proteins within healthy and high-risk populations.[12,14]

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In this study, we sought to discover biomarkers associated with OH by using a novel proteomic chip technology for the analysis of a multiplex protein panel of CVD biomarkers. Our aim was to identify a proteomic signature potentially useful to understand the pathophysiological pathway underlying the link between OH and incident cardiovascular morbidity, as observed in epidemiological studies.

Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

Study Population

The study was performed from September 2008 to May 2014 as a part of the SYSTEMA (Syncope Study of Unselected Population in Malmö).[10] Patients with unexplained syncope or symptoms of orthostatic intolerance were referred to the tertiary syncope unit at Skåne University Hospital in Malmö from outpatient care and hospitals in southern Sweden. Before examination at the syncope unit, patients underwent additional tests, such as exercise and ambulatory prolonged ECG, echocardiography, coronary angiography, brain imaging, and encephalography, if indicated, to exclude cardiac and neurological causes of symptoms. During the study period, a total of 994 patients were investigated with head-up tilt test (HUT) according to current European syncope guidelines[15]; of these, 778 patients had blood samples taken during their visit to the HUT laboratory (Figure 1).

Examination Protocol

Before examination, patients were asked to take their regular medications and fast for 2 hours but were allowed to drink water at will. Each patient completed a questionnaire concerning their medical history. The patients were placed on a tilt table and rested for at least 10 minutes before blood samples were collected through a venous cannula inserted in the forearm. Then, the patients rested for additional 10 minutes to achieve hemodynamically stable parameters; thereafter, the standardized 70° HUT was performed for 20 minutes followed by nitroglycerine provocation according to the Italian protocol, if passive HUT was negative, or until syncope/presyncope or pronounced symptoms of orthostatic intolerance occurred.[16] Continuous monitoring of beat-to-beat blood pressure (BP) and ECG was performed by a validated noninvasive photoplethysmographic method (Nexfin monitor; BMEYE, Amsterdam, The Netherlands) using a wrist unit and finger cuff of appropriate size.[17] All patients provided written informed consent. The Regional Ethical Review Board of Lund University approved the study protocol including blood sampling during tilt testing (No. 82/2008).

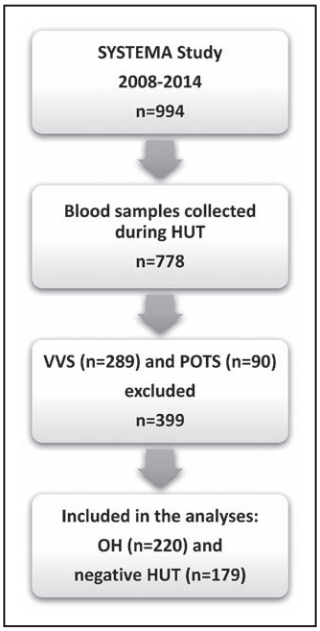

Figure 1. Flowchart of patient selection. HUT indicates head-up tilt; OH, orthostatic hypotension; POTS, postural orthostatic tachycardia syndrome; SYSTEMA, Syncope Study of Unselected Population in Malmö; and VVS, vasovagal syncope.

Proteomic Analysis

Plasma biomarkers were measured from supine blood samples (30 mL) that had been centrifuged, stored as 16×250 µL aliquots of EDTA plasma in plastic thermotubes, and frozen at −80°C after collection. The samples were thawed and examined by the Proximity Extension Assay technique using the Olink Proteomics Proseek Multiplex CVD I 96×96 reagents kit, which simultaneously measures 92 CVD-related human protein biomarkers in plasma. Briefly, a pair of oligonucleotide-labeled antibodies, Proseek probes, binds to the target protein in the plasma sample. When the 2 Proseek probes are in close proximity, a new polymerase chain reaction target sequence is formed by a proximity-dependent DNA polymerization event. This complex is subsequently detected and quantified using standard real-time polymerase chain reaction. The generated normalized protein expression unit is on a log2 scale where a larger number represents a higher protein level in the sample.

More details about limit of detection, reproducibility, and validation are available at the Olink Proteomics website (http://www.olink.com/products/document-download-center/).

Data Analysis

Supine BP and heart rate (HR) and lowest BP/highest HR during HUT were calculated as an average of a 30-second period. Supine values were obtained by analysis of a stable period of at least 1 minute before the start of HUT. During passive HUT, we analyzed and averaged the 30-second period before any of the following occurred: end of passive HUT with negative test result or test termination because of profound hypotension, pronounced symptoms or syncope.

OH was defined as a sustained decrease in systolic BP (SBP) ≥20 mm Hg or decrease in diastolic BP ≥10 mm Hg during passive HUT, with delayed OH being defined as significant BP fall occurring first after 3 minutes of HUT.[18] Vasovagal syncope was defined as a reproduction of syncope associated with a characteristic pattern of pronounced hypotension, bradycardia, or asystole,[15] whereas postural orthostatic tachycardia syndrome as a reproduction of symptoms of orthostatic intolerance (light-headedness, dizziness, or discomfort) with heart rate increase >30 per minute or tachycardia >120 per minute during HUT.[15,18] Body mass index was calculated by the Du Bois method.

Statistical Analysis

Patients with normal hemodynamic response and OH during passive HUT were included in the analyses. The main characteristics of study population are presented as mean and SD for continuous variables and as percentages for categorical variables. The baroreflex sensitivity (ms/mm Hg) index was calculated according to the formula (60/highest HR during HUT−60/supine HR)×1000 ms/(lowest SBP during HUT−supine SBP) and compared between OH-positive and OH-negative patients using nonparametric Mann–Whitney *U* test. The baroreflex sensitivity index was determined as the slope of the regression line of R-R intervals plotted against max change in SBP during HUT in patients with and without OH.

The discovery algorithm for the identification of potentially relevant biomarkers associated with the presence of OH was a sequential 2-step process of (1) biomarker signature identification by supervised, multivariate, principal component analysis and (2) verification by univariate ANOVA with Bonferroni correction.

After defining a minimal call rate <75%, we screened the CVD panel through supervised principal component analysis, according to the algorithm first described by Hastie and Tibsirani,[19] which includes the following steps:

1. For each proteomic maker, compute the standardized univariate logistic regression coefficient that represents the effect size for the outcome (presence or absence of OH);Using an arbitrary effect size threshold θ from the list $0 \le \theta_1 < \theta_2 < \ldots < \theta_K$,
2. Form a reduced data matrix consisting of only those proteomic markers whose univariate coefficient exceeds θ in absolute value, and compute the principal components of this matrix;Use these principal components in a multivariate logistic regression model to predict OH status;
3. Select the threshold θ that gives the best predictive accuracy by 10-fold cross-validation.

Thereafter, for the verification of the selected biomarkers, we applied a conservative univariate ANOVA approach, using a Bonferroni-adjusted significance level of P=0.05/11. Thus, the intergroup (OH+ versus OH−) difference was considered to be statistically significant with a P value <0.0045. Box plots were generated to display the distribution of biomarker levels between groups.

Furthermore, we performed univariate ordinary least square linear regression models for bivariate correlation between orthostatic SBP change (ΔSBP) and proteomic expression level and multivariate regression models adjusted for age, sex, supine SBP, diabetes mellitus, hypertension, antihypertensive treatment, prevalent CVD, and smoking.

Finally, we performed a quantile-regression analysis to identify differing relationships at different quartiles of SBP changes during HUT.

Statistical analyses were performed using IBM SPSS Statistics, version 23 (SPSS, Inc, Chicago, IL), and R Statistical Software (version 2.14.0; R Foundation for Statistical Computing, Vienna, Austria).

Results

Among 778 patients with available plasma samples (Figure 1), we identified 220 patients who met OH criteria and 179 patients with normal hemodynamic response during HUT (ie, no vasovagal syncope, no OH, and no postural orthostatic tachycardia syndrome). Among patients with OH, there were 99 with classical OH and 121 with delayed OH; of these, 4 with Parkinson disease, 5 with dementia, 1 with pure autonomic failure, and 1 with autoimmune autonomic ganglionopathy, whereas no primary neurodegenerative disease was found in the remaining group.

The baroreflex sensitivity index was clearly impaired in patients with OH (4±8 ms/mm Hg) compared with normal HUT response (12±32 ms/mm Hg; P<0.001; Figure S2 in the online-only Data Supplement). Patients with OH had a slightly greater increase in plasma norepinephrine level compared with those without OH (Δnorepinephrine, 1.22±1.05 versus 0.97±0.72 nmol/L; P=0.01). Descriptive characteristics of the study population are summarized in Table 1. For B-type natriuretic peptide (70%), β-nerve growth factor (56%), and interleukin-4 (0%), the call rate was below 75%, and these biomarkers were not included in the analysis.

Biomarker Signature Discovery

The data set consisted of 399 patients (220 OH and 179 controls). Because the principal component analysis requires pairwise complete data, all rows with missing data were removed. After removal of missing data, 225 patients remained (Table S1). Univariate logistic regression was performed for each of the 89 proteomic markers. The regression coefficients were then standardized by dividing the coefficient with its standard error. All possible thresholds (standardized coefficient (θ) ranging from minimum to maximum with 0.05 increment) were used to select groups of proteomic biomarkers and construct principal components (PCs). Two PCs from each group of biomarker were then regressed onto the outcome variable (OH status) using the binomial link function. This step identified the group of biomarkers that gave the best classification accuracy. The threshold that gave the best classification accuracy (OH+ versus OH−) was selected by 10-fold cross-validation. The following 11 proteomic markers (Table 2) reached this threshold: PAR-1 (proteinase-activated receptor-1), MB (myoglobin), MCP-1 (monocyte chemotactic protein-1), TM (thrombomodulin), PlGF (placenta growth factor), MMP-7 (matrix metalloproteinase-7), CASP-8 (caspase-8), TIM-1 (T-cell immunoglobulin and mucin domain-1), LOX-1 (oxidized low-density lipoprotein receptor-1), Dkk-1 (dickkopf-related protein-1), and CXCL-1 (C-X-C motif chemokine-1).

Table 1. Patient Characteristics According to OH Status

Characteristics	OH Positive (n=220)	OH Negative (n=179)	P Value
Age, y	66.5±15.9	59.8±20.4	<0.001
Sex (men), %	47.7	41.3	<0.001
Body mass index, kg/m^2	25.3±4.3	25.9±4.6	0.23
SBP supine, mm Hg	138.5±23.5	135.7±20.7	0.21
DBP supine, mm Hg	72.4±10.4	71.8±9.6	0.60
SBP HUT min, mm Hg	92.4±22.3	124.7±19.4	<0.001
DBP HUT min, mm Hg	58.5±12.6	72.3±10.9	<0.001
Heart rate supine, bpm	69.4±12.3	69.3±11.6	0.91
Heart rate HUT max, bpm	81.7±14.5	77.6±14.1	0.005
BRS index, ms/mm Hg	4±8	12±32	<0.001
Hypertension (n), %	50.7	40.2	0.26
Heart failure, %	5.9	6.7	0.74
Ischemic heart disease, %	12.3	11.2	0.31
Atrial fibrillation, %	6.9	8.4	0.68
Diabetes mellitus, %	10.0	9.0	0.56
Current smoking, %	12.2	16.5	0.47

P values for differences between the groups are shown as mean and SD for continuous variables and as percentages for categorical variables. BRS indicates baroreflex sensitivity; DBP, diastolic blood pressure; HUT min/max, lowest/highest value during passive head-up tilt test; OH, orthostatic hypotension; and SBP, systolic blood pressure.

Biomarker Verification

As shown in Table 3, of the selected 11 biomarkers, 8 proteins differed significantly between the groups in pairwise comparison univariate ANOVA. However, after Bonferroni correction, only 4 proteins met the predefined significance criteria: MMP-7 (P=0.00025), TIM-1 (P=0.00015), MB (P=0.001), and TM (P=0.00027). Distributions of plasma concentration of MMP-7, TM, MB, and TIM-1 by OH status are displayed in Figure 2. All 4 biomarkers were significantly associated with ΔSBP, even after Bonferroni correction for multiple testing at univariate regression analysis (Tables S2 and S3). In multivariate regression analysis, only MMP-7 and TIM-1 remained significantly associated with ΔSBP, even after Bonferroni

Table 2. Proteomic Signature by Supervised Principal Component Analysis: List of All Selected Biomarkers Associated With Orthostatic Hypotension and Their Biological Function

Biomarkers	Postulated Biological Mechanisms
MMP-7	Plasma levels of MMP-7 are increased in type-2 diabetes mellitus and linked to more severe atherosclerosis and coronary events. Thus, MMP-7 can play a role as a potential biomarker of atherosclerosis and increased cardiovascular disease risk.
TM	TM is an anticoagulant, and decreased levels can cause uncontrolled thrombus formation as a result of endothelial dysfunction of the atheromatous plaque. It can mediate both anti- and profibrinolytic effects depending on its concentration. Higher levels are found in patients with cardioembolic stroke and myocardial infarction.
MB	MB is one of the earliest biomarkers collected from patients suspected or diagnosed with cardiovascular disease. Increased levels of myoglobin indicate hemodynamic stress leading to myocardial microinjuries.
TIM-1	TIM-1 is expressed by activated T cells but is also found on dendritic cells and B cells and upregulated during renal injury. TIM-1 has been associated to plaque occurrence in carotid arteries in a human population–based study using proteomic arrays. Treatment with anti–TIM-1 (3D10) aggravates atherosclerosis, independent of cholesterol and triglyceride levels. Two important processes contributing to atherosclerosis development seem to be affected by TIM-1 blockade: efferocytosis and adaptive immune responses.
CASP-8	CASP-8 is involved in apoptosis and activation of lymphocytes T, B, and NK and macrophage differentiation. Decreased levels of CASP-8 have been linked to immunodeficiency.
CXCL-1	CXCL-1 is involved in inflammatory response and has been involved in several inflammation-related diseases, as well as in the processes of angiogenesis, arteriogenesis, and tumorigenesis.
Dkk-1	Dkk-1 is mainly associated with bone disease, cancer, and Alzheimer disease, and increased levels in bone marrow, plasma, and peripheral blood are seen in patients with multiple myeloma.
LOX-1	LOX-1 is a receptor that takes part in the process of oxidative modification of LDL. OxLDL is a marker of atherosclerosis that induces vascular endothelial cell activation and dysfunction, causing proinflammatory and pro-oxidative responses and apoptosis. Moreover, mutations in the gene encoding LOX-1 have been linked to atherosclerosis and myocardial infarction.
MCP-1	MCP-1 is a chemokine secreted by monocytes, macrophages, and dendritic cells and has been linked to atherosclerosis, neuroinflammation, and ulcerative colitis.
PAR-1	PAR-1 mediates the cellular effects of thrombin and contributes to thrombosis and the proinflammatory response observed in atherosclerosis and restenosis.
PIGF	PGIF has been related to angiogenesis and endothelial cell growth. Expression of PIGF within atherosclerotic lesions is associated with plaque inflammation.
TF	Higher levels are found in patients with myocardial infarction.

Adapted from Olink Proteomics website (www.olink.com/products/complete-biomarker-list/#). CASP-8 indicates caspase-8; CXCL-1, C-X-C motif chemokine-1; Dkk-1, dickkopf-related protein-1; LDL, low-density lipoprotein; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; MB, myoglobin; MCP-1, monocyte chemotactic protein-1; MMP-7, matrix metalloproteinase-7; OxLDL, oxidized low-density lipoprotein; PAR-1, proteinase-activated receptor-1; PIGF, placenta growth factor; TF, tissue factor; TIM-1, T-cell immunoglobulin and mucin domain-1; and TM, thrombomodulin.

Table 3. Verification of Principal Component Analysis Selection of 11 Cardiovascular Biomarkers in 399 Patients With Unexplained Syncope or Orthostatic Intolerance Stratified by OH Status

Biomarkers	OH Positive (n=220)	OH Negative (n=179)	*P* Value
CASP-8	1.56±0.66	1.46±0.64	0.15
CXCL-1	7.50±0.67	7.43±0.72	0.34
Dkk-1	6.20±0.55	6.10±0.54	0.12
LOX-1	4.62±0.54	4.50±0.57	0.03
MMP-7	6.75±0.69*	6.50±0.67*	<0.001*
MCP-1	3.59±0.59	3.50±0.51	0.03
MB	5.74±0.72*	5.50±0.72*	0.001*
PIGF	7.62±0.54	7.49±0.59	0.02
PAR-1	8.62±0.43	8.53±0.47	0.04
TIM-1	6.02±1.11*	5.60±1.11*	<0.001*
TM	9.67±0.38*	9.53±0.36*	<0.001*

Plasma concentrations of the assessed proteins are expressed on a log2 scale. Intergroup differences were assessed using ANOVA method. CASP-8 indicates caspase-8; CXCL-1, C-X-C motif chemokine-1; Dkk-1, dickkopf-related protein-1; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; MB, myoglobin; MCP-1, monocyte chemotactic protein-1; MMP-7, matrix metalloproteinase-7; OH, orthostatic hypotension; PAR-1, proteinase-activated receptor-1; PIGF, placenta growth factor; TIM-1, T-cell immunoglobulin and mucin domain-1; and TM, thrombomodulin.

*Bonferroni-corrected significant values ($P<0.0045$).

correction for multiple testing (Table 4). No obvious threshold effect or step function of MMP-7, TM, MB, and TIM-1 over quartiles of ΔSBP were observed by quantile-regression analyses (Figure S1).

Additional analyses showed that the norepinephrine response in classical OH did not differ from OH-negative patients (1.20±1.23 versus 0.97±0.72 nmol/L; $P=0.29$), in contrast to delayed OH (1.23±0.89; $P=0.03$). Nevertheless, both classical and delayed OH significantly differed from OH-negative patients with regard to selected biomarkers (Table S4).

Discussion

In this study, we observed that the presence of OH was associated with higher plasma concentration of several cardiovascular biomarkers, in particular of MMP-7, MB, TM, and TIM-1. These 4 biomarkers, validated by a more conservative univariate approach, were primarily selected by multivariate principal component analysis, which identified 7 other proteins as potentially related to OH.

Notably, MMP-7 and TIM-1 were found to be independently associated with orthostatic changes in SBP.

Recent technological advances enable simultaneous measurement of multiple plasma proteins. In this study, a novel state-of-the-art–targeted proteomics approach was used to explore the associations between circulating cardiovascular biomarkers and prevalent OH. This new technology has recently been used in many studies,[20] and our investigation adds methodological information in this developing field. The

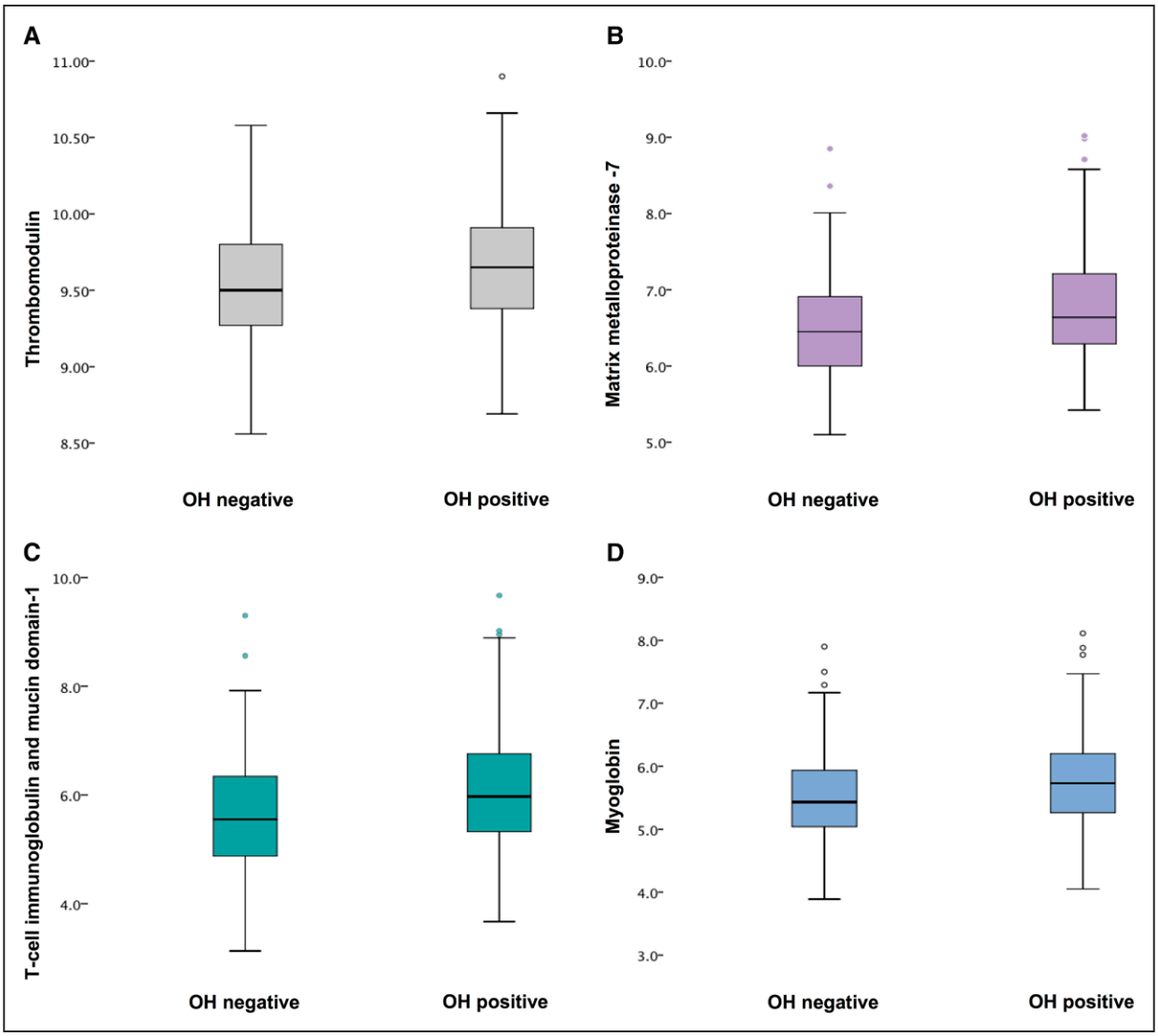

Figure 2. The plasma levels of (**A**) TM (thrombomodulin), (**B**) MMP-7 (matrix metalloproteinase-7), (**C**) TIM-1 (T-cell immunoglobulin and mucin domain-1), and (**D**) MB (myoglobin), expressed on a log2 scale presented in relation to orthostatic hypotension (OH) status. Data are shown as a box and whisker plot with median in the box and the whiskers representing the 5th and 95th percentiles in relation to plasmatic biomarker levels. Individual outlying values are displayed as circles.

broad-range screening used in this study seemed to be easily performed based on standard blood sample collection and provided multiple biomarker data that can be used both as a possible mechanistic explanation of observed associations, as well as a prognostic tool. Further, insights into the molecular background of OH may prove valuable for therapy tailoring in this understudied and difficult-to-treat condition.

The longitudinal relationship between OH and CVD has been consistently demonstrated in several independent population-based cohorts,[4,5,7,21,22] but the underlying mechanisms are not fully understood. Here, we provide evidence supporting the hypothesis that cardiovascular autonomic dysfunction underlying OH is not only a symptom-generating condition but also a disorder that has impact on important molecular mechanisms. The higher level of TM suggests a compensatory mechanism against a hypercoagulable state.[23] Higher MMP-7 indicates susceptibility to atherosclerosis, cardiac remodeling, and CVD,[13] while 2 important processes contributing to atherosclerosis development seem to be affected by TIM-1 blockade: efferocytosis and adaptive immune responses.[24] MB is increased in hemodynamic

Table 4. Association Between Changes in Systolic Blood Pressure During Head-Up Tilt Test and Proteomic Biomarkers in Multivariate Regression

Biomarkers	β Coefficient	95% CI	*P* Value
MMP-7	7.88†	3.75–12.01†	<0.001
TM	8.38	1.46–15.29	0.07
MB	2.78	–1.13 to 6.69	0.66
TIM-1	4.04†	1.23–6.85†	0.02

Multivariate analysis adjusted for age, sex, supine systolic blood pressure, diabetes mellitus, hypertension, antihypertensive treatment, presence of cardiovascular disease, and smoking. CI indicates confidence interval; MB, myoglobin; MMP-7, matrix metalloproteinase-7; TIM-1, T-cell immunoglobulin and mucin domain-1; and TM, thrombomodulin.

cardiac stress,[25] possibly associated with BP instability in OH and cardiac remodeling.

MMP-7

Studies have confirmed that MMPs (matrix metalloproteinases) are involved in matrix degradation within the atherosclerotic lesion, causing plaque destabilization and ischemic stroke.[26] It has been found that plasma levels of MMP-7 are increased in type-2 diabetes mellitus and linked to more severe atherosclerosis and coronary events. Thus, MMP-7 can play a role as a potential biomarker of atherosclerosis and increased CVD risk.[27]

TM

TM is an anticoagulant and scaffold for thrombin to activate protein C, leading to degradation of clotting factors Va and VIIIa, and to inhibition of the coagulation reactions and formation of fibrin.[23] Decreased levels of TM can cause uncontrolled thrombus formation as a result of endothelial dysfunction of the atheromatous plaque.[23] Moreover, TM is involved in the regulation of fibrinolysis and can mediate both anti- and profibrinolytic effects depending on its concentration.[28] Higher levels of TM were found in patients experiencing an embolic stroke.[29] Interestingly, both TM and tissue factor are increased in myocardial infarction.[30] Further, shedding of TM from dysfunctional endothelium has been associated to the attenuation of anticoagulant activities of protein C within the atherosclerotic plaque likely promoting atherothrombotic events.[31]

MB

MB is largely accepted as one of the earliest biomarkers routinely collected from patients suspected or diagnosed with CVD.[32] Increased levels of MB indicate hemodynamic stress leading to myocardial microinjuries.[25]

TIM-1

TIM-1 is expressed on activated T cells and regulatory B cells and might have an important role in regulating immune responses. Recently, in a human population–based study using proteomic arrays, Lind et al[12] found an association between Tim-1 and plaque occurrence in carotid arteries.

Other Potential Biomarkers

Supervised principal component analysis is an effective dimensionality-reduction technique useful to select relevant proteomic biomarkers, by taking into consideration the correlation structure of the data and the interactions existing among the potential biomarkers and generally ensuring diagnostic and prognostic performances superior to single markers in terms of sensitivity, specificity, and reliability.[33] By this method, we were able to discover a cluster of other 7 proteomic biomarkers associated with OH and linked to some extent with vascular inflammation and atherothrombosis (Table 3).

General Remarks

The observed associations support the hypothesis that OH and cardiovascular autonomic dysfunction are not linked to 1 mechanism promoting CVD but that this relationship is multifactorial, including hypercoagulability, endothelial dysfunction, vascular inflammation, prolonged myocardial damage, and atherothrombosis. Increased levels of MMP-7, TM, MB, and TIM-1 are in line with previous observations that presence of OH leads to premature atherosclerosis, systemic inflammation,[34] elevated plasma concentration of von Willebrand factor,[10] and left ventricular hypertrophy.[9] Notably, increased endothelin-1 production has been reported as a proxy for postural hemodynamic instability, likely counteracting the hypotensive tendency during orthostasis in patients with OH.[35] Endothelin-1 is also involved in the activation of transcription factors, such as NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), and expression of proinflammatory cytokines.[36] Further, prolonged orthostatic stress is known to trigger the coagulation cascade through endothelial activation and reduction in the global activity of protein C pathway, even in healthy subjects, introducing the concept of a relatively novel physiological mechanism of orthostatic hypercoagulation.[34,37] Interestingly, accumulating data would suggest an intriguing extensive bidirectional cross-talk between impaired hemostasis and inflammation, which are both downstream events regulated by OH, in the pathophysiology of many complex diseases, including atherothrombosis.[31]

This report adds to the growing evidence that orthostatic BP instability is not only a hemodynamic phenomenon but includes a range of dysregulated molecular events with potentially detrimental impact on the circulatory homeostasis. However, the cross-sectional design of the study does not allow exclusion of a reverse causality: the association between markers of atherosclerosis and OH may indicate a prevalent disease of the arteries that prevents stretch of the baroreceptors in response to changes in BP, thus leading to a diminished autonomic response to upright tilt because of poor baroreceptor input to the brain stem. Finally, the chronotropic response on HUT was more pronounced in the OH group compared with normal HUT response (+12 versus +8 bpm), suggesting that the cardiovagal control was preserved to some extent, although insufficient to provide an effective homeostatic control of circulation during orthostasis. The chronotropic insufficiency was well illustrated by the impaired baroreflex sensitivity in the OH group. However, impaired norepinephrine response was mainly observed in classical OH.

Strengths and Limitations

The strengths of this study include the use of a novel targeted proteomics chip and a rather large number of patients with symptomatic orthostatic intolerance. Furthermore, all patients were examined according to a standardized protocol with beat-to-beat hemodynamic monitoring and extended observational period, thereby reducing the risk of inaccurate or missed diagnosis of OH.[10] Finally, we performed a sequential 2-step discovery and verification analysis, the former based on a supervised, multivariate, dimensionality-reduction technique, achieving the best compromise between best predictive ability and exhaustivity, and the latter using a more conservative approach through univariate ANOVA with Bonferroni adjustment.[33]

There are some limitations that should be addressed. In the laboratory setting of HUT provocation test, the acute psychosomatic stress related to the test performance may have strengthened the prothrombotic effects by increased sympathetic activity. We acknowledge the lack of a thorough evaluation of the autonomic nervous system function. In particular, the integrity of the cardiovagal baroreflex function, the changes in carotid artery mechanics during HUT, and the specific association between markers of autonomic function, that is, neuroendocrine markers, power spectral analysis of HR, or BP variability, and selected CVD biomarkers have not been explored but should be the object of future investigations aimed to understand the biological processes linking autonomic dysfunction to the CVD continuum.

The study was performed on a series of symptomatic individuals who were not aware of the nature of underlying disorder before investigation. Thus, the study may not be fully representative of OH detected in the general population through the screening programs, and the generalizability to other age, race, and ethnic groups is unknown.

Perspectives

Our study supports and extends the concept that proteomic profiling may considerably improve the understanding of the mechanisms by which OH is associated with increased cardiovascular morbidity and mortality. We observed that the presence of OH in patients with history of unexplained syncope and orthostatic intolerance is associated with a biomarker signature linked to vascular inflammation and atherothrombosis and, in particular, with higher plasma concentrations of MMP-7, TM, and MB. These findings are in keeping with the hypothesis of pleiotropic effects evoked by cardiovascular autonomic dysfunction on the circulatory system. Further research is warranted to confirm our preliminary findings in larger outcome studies.

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Disclosures

None.

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Novelty and Significance

What Is New?

- This is the first study to explore the cardiovascular proteomic signature in patients with orthostatic hypotension to unveil specific pathobiological mechanisms linking orthostatic hypotension and autonomic dysfunction with increased risk of cardiovascular disease.

What Is Relevant?

- Proteomic profiling in orthostatic hypotension reveals a biomarker signature of atherothrombosis and inflammation.
- Circulating levels of MMP-7 (matrix metalloproteinase-7), TM (thrombomodulin), MB (myoglobin), and TIM-1 (T-cell immunoglobulin and mucin domain-1) are strongly associated with orthostatic hypotension and may be involved in cardiovascular disease promotion.

Summary

A novel targeted proteomics approach using the proximity extension technique discovered several new associations of cardiovascular disease biomarkers with orthostatic hypotension prevalence. Orthostatic blood pressure instability is not only a hemodynamic phenomenon but includes a range of dysregulated molecular events yielding a prothrombotic and proinflammatory milieu with potentially detrimental impact on the cardiovascular homeostasis.

Proteomic Profiling for Cardiovascular Biomarker Discovery in Orthostatic Hypotension

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Online Supplementary Appendix

Proteomic profiling for cardiovascular biomarker discovery in orthostatic hypotension

Short title: Cardiovascular proteomic signature in OH

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Table S1.

Comparison of patient characteristics between the whole cohort and PCA cohort.

Characteristics	OH+			OH-		
	Overall	PCA cohort	P	Overall	PCA cohort	P
n	220	127		179	98	
Age (years)	66.5 (15.9)	68.9 (14.8)	ns	59.8 (20.4)	64.9 (18.0)	**0.04**
Sex (%, male)	105 (47.7)	60 (47.2)	ns	74 (41.3)	37 (37.8)	ns
Body-mass index (kg/m^2)	25.4 (4.3)	25.5 (4.4)	ns	25.9 (4.6)	26.2 (4.8)	ns
SBP supine (mmHg)	138.5 (23.5)	141.1 (23.6)	ns	135.7 (20.7)	139.1 (20.0)	ns
DBP supine (mmHg)	72.4 (10.4)	73.2 (9.5)	ns	71.8 (9.6)	72.4 (9.3)	ns
HR supine (bpm)	69.4 (12.3)	70.1 (13.1)	ns	69.3 (11.6)	69.9 (11.2)	ns
SBP HUT min(mmHg)	92.4 (22.3)	91.1 (22.6)	ns	124.7 (19.4)	126.7 (19.6)	ns
DBP HUT min (mmHg)	58.5 (12.6)	57.9 (12.3)	ns	72.3 (10.9)	72.1 (10.4)	ns
HR HUT max (bpm)	81.7 (14.5)	80.6 (14.6)	ns	77.6 (14.1)	76.9 (12.6)	ns
Hypertension (n, %)	110 (50.7)	71 (57.3)	ns	72 (40.2)	47 (48.0)	ns
IHD (%)	27 (12.3)	15 (11.9)	ns	20 (11.2)	14 (14.3)	ns
Heart failure (%)	13 (5.9)	8 (6.3)	ns	12 (6.7)	5 (5.1)	ns
Atrial fibrillation (%)	15 (6.9)	10 (8.0)	ns	15 (8.4)	10 (10.3)	ns
Diabetes mellitus (%)	22 (10.0)	12 (9.4)	ns	16 (9.0)	11 (11.3)	ns
Current smoking (%)	18 (8.3)	9 (7.1)	ns	35 (19.6)	19 (19.4)	ns

P values for differences between the groups shown as mean and standard deviation for continuous variables and as percentages for categorical variables. OH, orthostatic hypotension; SBP, systolic blood pressure; DBP, diastolic blood pressure; HUT min/max, lowest/highest value during passive head-up tilt test; bpm, beats per minute; IHD, ischemic heart disease; PCA, principal component analysis.

Table S2.

Association between delta SBP and proteomic biomarkers in univariate regression.

Biomarker	Regression coefficient	95% CI	Bonferroni-corrected P-value
MMP-7	10.57	7.00 - 14.13	< 0.0001
TM	16.25	9.66 - 22.83	< 0.0001
MB	7.85	4.44 - 11.26	< 0.0001
TIM-1	6.69	4.52 - 8.86	< 0.0001

Table S3.

Association between delta SBP with no extreme values and proteomic biomarkers in univariate regression.

Biomarker	Regression coefficient	95% CI	Bonferroni-corrected P value
MMP-7	8.67	5.55 - 11.78	< 0.0001
TM	14.53	8.81 - 20.24	< 0.0001
MB	6.95	4.00 - 9.91	< 0.0001
TIM-1	5.14	3.22 - 7.06	< 0.0001

Table S4.

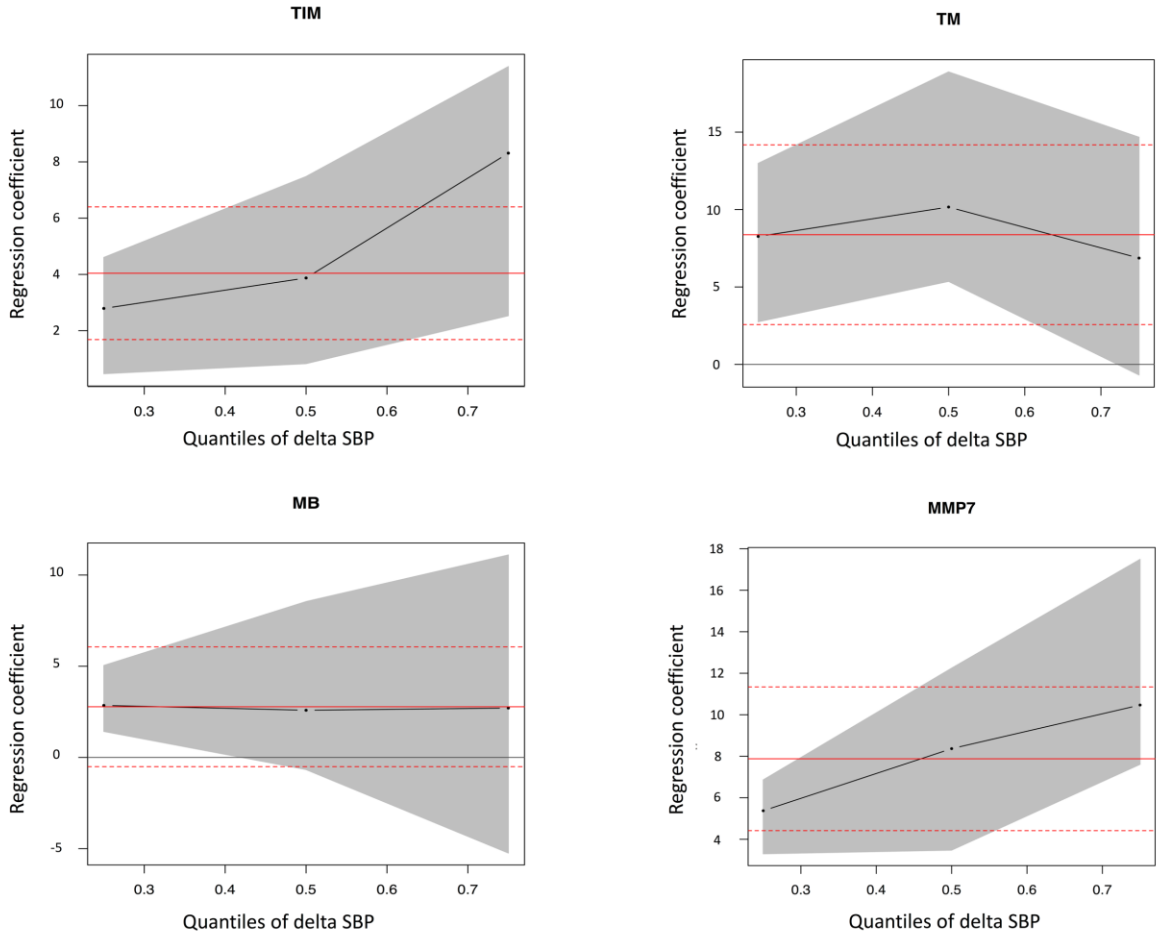
Comparison between classical and delayed OH vs. OH-negative patients.

Variable	OH negative (n=179)	Classical OH (n=99)	Delayed OH (n=121)	p-value (Classical OH vs OH negative)	p-value (Delayed OH vs OH negative)
Δ RR interval (ms)	92.25 (91.86)	121.88 (93.06)	138.15 (133.62)	0.02	0.004
Δ Norepinephrine (nmol/L)	0.97 (0.72)	1.20 (1.23)	1.23 (0.89)	0.19	0.03
Matrix metalloproteinase-7 (MMP-7)*	6.50 (0.67)	6.81 (0.68)	6.71 (0.70)	0.001	0.02
Thrombomodulin (TM)*	9.53 (0.36)	9.68 (0.40)	9.65 (0.37)	0.005	0.008
Myoglobin (MB)*	5.49 (0.72)	5.72 (0.70)	5.75 (0.74)	0.02	0.01
T-cell immunoglobulin and mucin domain-1 (TIM-1)*	5.59 (1.11)	6.00 (1.04)	6.03 (1.16)	0.005	0.004

Δ RR = supine RR – max. RR on standing; Δ Norepinephrine = standing norepinephrine – supine norepinephrine; All continuous data are presented in mean (SD); *Biomarkers are expressed in an arbitrary unit on Log2 scale.

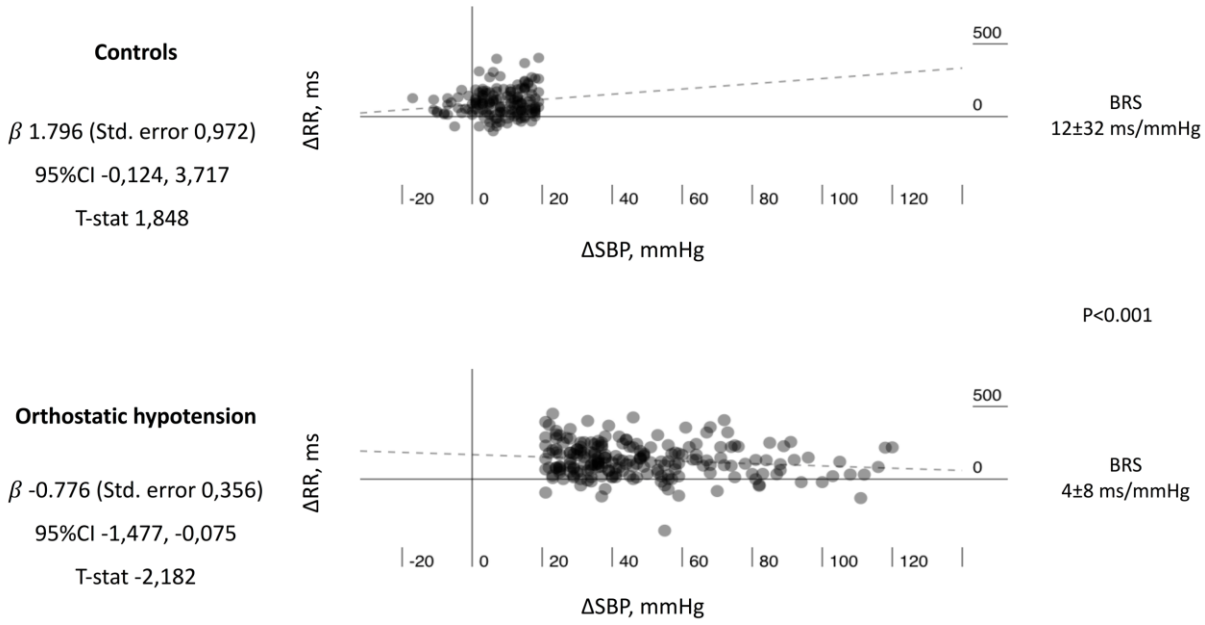
Figure S1.

Adjusted quantile regression analysis.



Adjusted quantile regression analysis. Each biomarker (adjusted for age, sex, supine systolic blood pressure, diabetes mellitus, hypertension, antihypertensive treatment, prevalent cardiovascular disease and smoking) regressed on 25th, 50th and 75th quantiles of delta SBP. The x axis is the quantile of delta SBP (black dots in the plots represent the regression coefficient at 0.25, 0.5 (median) and 0.75). The grey bands are the 95% CI of the quantile regression coefficient. The horizontal red and the two horizontal dotted lines are the ordinary least square (OLS) linear regression lines. What you can see here is that 95% CI of the coefficients from quantile regression overlaps widely with OLS lines indicating that the biomarkers do not have differing effects on different quantiles of delta SBP.

Figure S2.



Baroreflex sensitivity according to OH status. Baroreflex sensitivity assessed by mean index (mean ratio of all RR interval changes and systolic blood pressure changes, ms/mmHg) and linear regression of change in R-R interval with change in systolic blood pressure) in patients with orthostatic hypotension and in patients with normal haemodynamic response on head-up tilt test. In the graph the slopes of the regression lines run in opposite directions, with minimal overlap between the 95%CIs of the beta regression coefficients. BRS, baroreflex sensitivity; CI, confidence interval.