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Abstract

Objective: To investigate the inflammatory proteomic signature associated with classical orthostatic hypotension. Methods: A cross-sectional study including 778 patients with unexplained syncope and/or orthostatic intolerance undergoing head-up tilt test (HUT) and supine blood sampling. Of these, 98 met diagnostic criteria of classical OH and 181 demonstrated normal haemodynamic response during HUT. Blood samples were analysed by antibody-based Proximity Extension Assay technique simultaneously measuring 57 inflammatory and cancer-related human protein biomarkers. The discovery algorithm was a sequential two-step process of biomarker signature identification by multivariate principal component analysis (PCA), and verification by univariate ANOVA with Bonferroni correction. Results: Patients with classical OH were older (68 vs. 60 years; $p<0.001$) and more likely to be men (58 vs. 41%; $p<0.001$). PCA and Bonferroni-adjusted ANOVA identified midkine (MK), immunoglobulin-like transcript 3 (ILT-3), regenerating islet-derived protein 4 (REG-4), and tartrate-resistant acid phosphatase type 5 (TR-AP) as the most robust proteomic signature for OH. In multivariate regression analysis adjusting for age, sex, cardiovascular disease and risk factors, the results remained significant for ILT-3 ($p=0.036$), MK ($p=0.008$) and REG-4 ($p=0.024$), but not for TR-AP. Conclusions: Proteomic profiling in classical orthostatic hypotension reveals a biomarker signature associated with immunoregulatory functions and vascular inflammation. Circulating levels of midkine, immunoglobulin-like transcript-3, regenerating islet-derived protein-4 are elevated in orthostatic hypotension, suggesting a complex interplay amongst inflammation, autonomic dysfunction and atherothrombosis.

Keywords	dysautonomia; inflammation; proteomics; autonomic function; cardiovascular system pathology.
Taxonomy	Autonomic Function, Proteomics, Inflammation, Cardiovascular System Pathology, Dysautonomia
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Data will be made available on request

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November 4th, 2017

Dear Professor Paolo G. Camici,

Please find enclosed the original research article:

"Proteomic signature of inflammation in classical orthostatic hypotension"

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here submitted to your attention for possible publication in the **International Journal of Cardiology**.

In this paper, we explored the possible mechanisms underlying the reciprocal interaction between autonomic dysfunction and inflammation - the so called central inflammatory reflex - by a proteomic approach in a cohort of 98 patients with classic orthostatic hypotension and 181 controls with normal hemodynamic response on head-up tilt testing. Interestingly, the proteomic profiling in orthostatic hypotension revealed a biomarker signature associated with immunoregulatory functions and vascular inflammation. In particular, circulating levels of midkine, immunoglobulin-like transcript-3, and regenerating islet-derived protein-4 have been found significantly elevated in patients with orthostatic hypotension, suggesting a complex interplay among inflammation, autonomic dysfunction and atherothrombosis.

We believe that the novelty of these findings would appeal the broad readership of your journal.

As a Corresponding Author, I declare, on behalf of the co-authors, that:
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2) none of the paper's contents have been previously published;
3) all authors have read and approved the manuscript;
4) the manuscript includes a full disclosure of any potential conflict of interest.

All authors have agreed on the submission of this manuscript to the **International Journal of Cardiology.**

We hope you will find our manuscript suitable for publication and look forward to hearing from you.

Sincerely yours,

Artur Fedorowski

HIGHLIGHTS

- Dysautonomia and inflammation have multifaceted and reciprocal interactions.
- OH revealed a proteomic signature of inflammation and immunoregulatory functions.
- Inflammation and autonomic dysfunction are likely intertwined in atherothrombosis.

Proteomic signature of inflammation in classical orthostatic hypotension

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The authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

The authors declare no conflict of interest.

Abstract

Objective: To investigate the inflammatory proteomic signature associated with classical orthostatic hypotension.

Methods: A cross-sectional study including 778 patients with unexplained syncope and/or orthostatic intolerance undergoing head-up tilt test (HUT) and supine blood sampling. Of these, 98 met diagnostic criteria of classical OH and 181 demonstrated normal haemodynamic response during HUT. Blood samples were analysed by antibody-based Proximity Extension Assay technique simultaneously measuring 57 inflammatory and cancer-related human protein biomarkers. The discovery algorithm was a sequential two-step process of biomarker signature identification by multivariate principal component analysis (PCA), and verification by univariate ANOVA with Bonferroni correction.

Results: Patients with classical OH were older (68 vs. 60 years; p<0.001) and more likely to be men (58 vs. 41%; p<0.001). PCA and Bonferroni-adjusted ANOVA identified midkine (MK), immunoglobulin-like transcript 3 (ILT-3), regenerating islet-derived protein 4 (REG-4), and tartrate-resistant acid phosphatase type 5 (TR-AP) as the most robust proteomic signature for OH. In multivariate regression analysis adjusting for age, sex, cardiovascular disease and risk factors, the results remained significant for ILT-3 (p=0.036), MK (p=0.008) and REG-4 (p=0.024), but not for TR-AP.

Conclusions: Proteomic profiling in classical orthostatic hypotension reveals a biomarker signature associated with immunoregulatory functions and vascular inflammation. Circulating levels of midkine, immunoglobulin-like transcript-3, regenerating islet-derived protein-4 are elevated in orthostatic hypotension, suggesting a complex interplay amongst inflammation, autonomic dysfunction and atherothrombosis.

Keywords: dysautonomia; inflammation; proteomics; autonomic function; cardiovascular system pathology.

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Introduction

Orthostatic hypotension (OH) is a hallmark sign of autonomic failure frequently observed in patients with neurodegenerative diseases and comorbidities, such as diabetes and hypertension[1-3]. Presence of OH may cause debilitating symptoms and indicates higher risk of cardiovascular disease (CVD) and premature death.[2, 4, 5] Nevertheless, OH is frequently overlooked in cardiovascular screening programmes, epidemiological studies, and diagnostic work-up of patients with symptoms potentially related to this condition[2].

Traditionally, OH is divided into two main categories: neurogenic and non-neurogenic[2]. Neurogenic OH is a primary manifestation of chronic autonomic failure in neurodegenerative disorders, such as pure autonomic failure, multiple system atrophy and Parkinson disease[6]. Orthostatic hypotension can also be secondary to various inflammatory and non-inflammatory conditions such as multiple myeloma, paraneoplastic syndrome, autoimmune diseases or amyloidosis[4], with a presumable affection of autonomic nervous system, although in many cases the aetiology remains unknown[1]. On the other hand, non-neurogenic OH can be caused by conditions that impair the compensatory mechanisms governed by the autonomic nervous system, such as diabetes and chronic cardiovascular disorders[2], but overlap between neurogenic and non-neurogenic factors in secondary OH may exist[2].

The most severe form of OH, often referred to as classical[7], implies a significant blood pressure reduction within the first three minutes of upright standing[8]. The majority of cases related to neurogenic OH belong to this category[2, 7]. However, in at least one third of cases, the aetiology of OH remains elusive, even after an extensive diagnostic work-up[1].

Both neurogenic and non-neurogenic forms of OH may potentially involve activation of inflammatory pathways, as components of the underlying pathological process eventually leading to autonomic failure[9]. Notably, a cholinergic anti-inflammatory

pathway that reflexively adjusts macrophage activation via parasympathetic outflow has recently been described[10]. Further, the immune system has been shown to modulate autonomic activity, hence completing the wiring of the so called "inflammatory reflex"[11]. Thus, it is important to explore the expression of inflammatory mediators in OH as a potential diagnostic tool and therapeutic target in this understudied and difficult-to-treat condition.

To this aim, we applied a novel proteomic chip technology to assess a wide panel of inflammatory and oncological biomarkers in patients with classical OH and in subjects with normal haemodynamic response during controlled orthostatic challenge.

Methods

Study population

The study was carried out from September 2008 to May 2014 as a part of the Syncope Study of Unselected Population in Malmö (SYSTEMA)[12]. Patients with unexplained syncope and/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. Additional tests were performed, if indicated, to eliminate any cardiac and neurological causes of the symptoms, e.g. exercise and ambulatory prolonged electrocardiogram (Holter ECG), 2D transthoracic echocardiography, coronary and pulmonary angiography, brain imaging and encephalography. During the study period, 994 patients were examined by head-up tilt test (HUT) according to current European syncope guidelines [7]; of these, 778 patients had blood samples collected during HUT examination (Fig.1). All patients gave written informed consent. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and has been approved by The Regional Ethical Review Board of Lund University (No 82/2008).

The PICO model was as follows: patients with unexplained syncope or orthostatic intolerance (Population), blood samples and HUT (Intervention), classical OH versus controls (Comparison), proteomic signature and hemodynamic response (Outcome).

Examination protocol

Patients were taking their regular medications, fasted for two hours prior to examination but were allowed to drink water at will. They were asked to fill out a questionnaire about past medical history. The patients were placed on a tilt table and rested for at least ten minutes before blood samples were collected through a venous cannula inserted in the forearm. Subsequently, patients rested for another 10 minutes to obtain haemodynamically stable parameters; thereafter the standardized 70°HUT was carried out for 20 minutes followed by nitroglycerine provocation according to the Italian protocol if passive HUT was negative, or until syncope/pre-syncope or pronounced symptoms of orthostatic intolerance occurred[13]. Beat-to-beat blood pressure and ECG was monitored continuously by a validated non-invasive photoplethysmographic method (Nexfin monitor; BMEYE, Amsterdam, Netherlands) with a wrist unit and finger cuff of appropriate size [14].

Proteomic analysis

Plasma biomarkers were measured from supine blood samples (total volume: 30 ml) that had been first centrifuged, then stored as 16x250 µL aliquots of EDTA plasma in plastic thermotubes, and frozen at -80° C. For biomarker analysis, the samples were thawed and examined by the Proximity Extension Assay technique using the Olink Proteomics Proseek Multiplex Oncology I v1 96x96 reagents kit, which simultaneously measures 57 inflammatory and cancer-related human protein biomarkers in plasma (Table S1). In short, a pair of oligonucleotide-labelled antibodies, Proseek probes, binds to the target protein in the plasma sample. When the two Proseek probes are in

close proximity, a new polymerase-chain reaction (PCR) target sequence is formed by a proximity-dependent DNA polymerization event. This complex is subsequently detected and quantified using standard real-time PCR. The generated Normalized Protein Expression (NPX) unit is on a log2 scale, which means that a larger number represents a higher protein level in the sample. Additional information about limit of detection, reproducibility and validation is available at the Olink Proteomics website (http://www.olink.com/products/document-download-center).

Data analysis

Supine and 3-min HUT BP were calculated over an averaged 30-second period. The supine BP was calculated during a stable period between 1 and 5 minutes prior to HUT. The 3-min HUT value was calculated after 3 min of HUT.

We defined classical orthostatic hypotension is a sustained drop in systolic BP ≥20 mm Hg and/or drop in diastolic BP (DBP) ≥10 mm Hg after 3 min of passive HUT[8]. A significant drop in BP occurring after 3 min of HUT was defined as delayed OH[15], and these patients were excluded from the analyses. Vasovagal syncope (VVS) was defined as a reproduction of syncope associated with a characteristic pattern of pronounced hypotension, bradycardia or asystole[7], while postural orthostatic tachycardia syndrome (POTS) as a reproduction of symptoms of orthostatic intolerance (lightheadedness, dizziness or discomfort) with heart rate increase >30/min or tachycardia >120/min during HUT[7, 8]. Patients with VVS and POTS were excluded from the analyses.

The baroreflex sensitivity (BRS; ms/mmHg) index was calculated according to the formula: (60/highest HR during HUT - 60/supine HR) x 1000 ms / (lowest SBP during HUT - supine SBP), and compared between OH-positive and OH-negative patients.

Valsalva maneuver was also performed to further assess nonpostural hemodynamic responses and BRS. Adrenergic BRS failure was featured by clear V-shaped SBP response, as previously reported[16].

We used the Modification of Diet in Renal Disease (MDRD) study equation to calculate the glomerular filtration rate.

Statistical analysis

The main characteristics of study population are presented as mean and standard deviation for continuous variables and as percentages for categorical variables.

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

After defining a minimal call rate <75%, we screened the proteomic panel through supervised principal component analysis, according to the algorithm first described by Hastie and Tibsirani[17], which includes the following steps:

1) For each proteomic maker, compute the standardized univariate logistic regression coefficient which represents the effect size for the outcome (presence or absence of OH);
2) Using an arbitrary effect size threshold θ from the list $0 \leq \theta_1 < \theta_2 < \cdots < \theta_K$:
    a. 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;
    b. Use these principal components in a multivariate logistic regression model to predict OH status;
3) Select the threshold θ which 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=0.05/4. Thus, the inter-group (OH+ vs. OH-) difference was considered to be statistically significant with a p-value <0.0125. 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 plasma level of selected biomarkers, and multivariate regression models adjusted for age, sex, supine systolic blood pressure, diabetes mellitus, hypertension, antihypertensive treatment, glomerular filtration rate, prevalent cardiovascular disease and smoking. Finally, we performed a quantile-regression analysis in order to identify differing relationships at different quartiles of SBP changes during HUT.

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

Results

Of 778 patients with available plasma samples (Fig.1), we found 98 patients who met classical OH criteria, and 181 patients with normal haemodynamic response during HUT. Descriptive characteristics of the study population are shown in Table 1. Four biomarkers were excluded from the analysis because their call rate was below 75%: erythropoietin (18%), interleukin-2 (7.9%), interferon-gamma (65%) and tumour necrosis factor (6.5%).

Biomarker signature discovery

The dataset consisted of 279 patients (98 OH and 181 controls). Since the principal component analysis requires pairwise complete data, we did not include markers with

high missingness (>5%). This filter resulted in removal of 4 biomarkers (vascular endothelial statin, lipopolysaccharide-induced tumour necrosis factor-alpha factor, MHC class I polypeptide-related sequence A and carcinoembryonic antigen). After removal of all missing data, 262 patients remained. Univariate logistic regression was performed for each of the 49 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 increments) were used to select groups of proteomic biomarkers and construct principal components (PCs). The outcome variable (OH status) were then regressed onto the first two PCs from each group of biomarkers using the binomial link function. This step identified the group of biomarkers which gave the best classification accuracy. The threshold that gave the best classification accuracy (OH+ vs OH-) was selected by ten-fold cross-validation. The following 4 proteomic markers reached this threshold: midkine (MK), immunoglobulin-like transcript 3 (ILT-3), regenerating islet-derived protein 4 (REG-4), and tartrate-resistant acid phosphatase type 5 (TR-AP).

Biomarker verification

As shown in Table 2, all PCA selected biomarkers differed significantly in pairwise comparison, even after Bonferroni correction. In multivariate regression analysis adjusting for age, sex, cardiovascular disease and risk factors, ΔSBP was still significantly associated with ILT-3 (p=0.036), MK (p=0.008) and REG-4 (p=0.024), but TR-AP did not reach statistical significance (Table 3).

Quantile regression analyses investigating the relationships between ILT-3, MK, REG-4 and TR-AP and the quartiles of ΔSBP did not reveal any obvious threshold effect or step function (Fig. S1).

Discussion

This study demonstrates that patients with orthostatic hypotension have elevated plasma levels of several inflammatory biomarkers, particularly immunoglobulin-like transcript 3 (ILT-3), midkine (MK) and regenerating islet-derived protein 4 (REG-4), independently of age, sex, prevalent cardiovascular disease and risk factors.

Thanks to recent technological advances it is possible to measure multiple plasma protein simultaneously. In this study, we applied a novel state-of-the-art targeted proteomics chip to investigate circulating inflammation and cancer-related proteins and their association with OH. Lately, the proteomics technology has been implemented in a number of studies[18], and this study adds further insights to this emerging field. Additional understanding of the molecular basis of OH may be of clinical importance in order to improve and personalize therapy in this understudied and difficult to treat condition.

Traditionally, OH has been linked to neurodegenerative diseases and chronic inflammatory conditions, more recently it has been found to be a common finding among patients with hypertension and diabetes.[2]

However, the relationship between OH and inflammatory responses have not been sufficiently explored. In this study, we provide evidence supporting the view that autonomic dysfunction underlying OH is not merely a symptom-generating condition, but also a disorder that has complex interplay with important inflammatory and immunological processes.

The elevated levels of MK suggest an acute cytoprotective effect in ischaemia/reperfusion injury related to its anti-apoptotic effect promoting angiogenesis and inhibition of cardiac tissue remodeling.[19] Moreover, MK facilitates endothelial cell proliferation, and also recruits inflammatory cells to the walls of the vessels promoting neointima formation, vascular stenosis and inflammation, inducing features of plaque vulnerability in atherosclerosis. Upregulation of ILT-3 seems to play a

significant role in graft adaptation and protection against the recipient's immune response.[20] Expression of REG-4 is considerably upregulated during inflammation and tissue injury associated with autoimmune diseases, such as active Crohn's disease and ulcerative colitis, and in colorectal cancer.[21] These findings have not been reported previously in OH patients.

Midkine

MK is a heparin-binding growth factor of low molecular weight involved in the aetiology of inflammatory diseases, e.g. multiple sclerosis.[22, 23] It is activated during oncogenesis, inflammation and tissue repair, and enhances cell proliferation, cell migration, angiogenesis and fibrinolysis. Elevated levels of MK are observed in several malignant tumours and it is also linked to tumour resistance to chemotherapeutics. Additionally, deposits of MK are seen in patients with neurodegenerative diseases, e.g. Alzheimer's disease and multiple system atrophy.[24]

Moreover, results published by Horiba et al.[25] suggest that MK may play a protective role against ischaemia/reperfusion injury and constitutes a new potentially important molecular target for treatment of ischaemic heart disease. Interestingly, MK is induced in cancer tissues where it also promotes angiogenesis and tumour formation by angiogenic and anti-apoptotic activity.[19] Muramatsu et al.[22] found that MK may be useful as a cancer marker, whereas MK itself may be used in treatment of brain and heart diseases. On the other hand, MK-inhibitors can be used in the treatment of malignant tumours, multiple sclerosis, restenosis, renal diseases, and hypertension.

In the heart, preclinical data support a potential role of MK in the pathophysiology of CVD, where it promotes endothelial cell proliferation and enhances plaque infiltration of inflammatory cells. Notably, MK-deficient mice exhibited significantly lower neointimal formation[26], while systemic administration of MK in apolipoprotein-E knockout mice increased atherosclerosis[27]. Recently, it has also been demonstrated

that MK could be used in humans to predict the presence of significant coronary artery disease and higher incidence of acute coronary events[28]. Taken together, these findings suggest that MK mediates atherosclerotic plaque formation and progression associated with a pro-inflammatory drive.

Immunoglobulin-like transcript 3

Immunoglobulin-like transcripts (ILTs) are immuno-regulatory proteins that either activate or inhibit immune responses.[29] ILT-3 is an important mediator of the induction of immune tolerance and expressed on monocytes and antigen-presenting cells such as macrophages and dendritic cells. Although the mechanisms by which ILT3 modulates immune responses is largely unknown, Chang et al. found that down-regulation of ILT3 may result in autoimmune diseases due to excess inflammation and infiltration of T cells in locally affected lesions.[30] Furthermore, studies of human heart transplant recipients demonstrated that rejection-free patients have circulating T-suppressor cells, which cause up-regulation of ILT3 in donor antigen-presenting cells. These results indicate a possibly important mechanism of immune regulation.[20]

Regenerating islet-derived protein 4

REG-4 is associated with inflammatory and metaplastic responses of the gastrointestinal epithelium. It is a critical protein involved in the development of colorectal cancer and overexpression of REG-4 with or without overexpression of matrix metalloproteinase 7 (MMP-7) is a predictive factor of poor prognosis in colorectal cancer.[31] It has been found that REG-4 promotes the proliferation and invasiveness of cancer cells by upregulating the expression of MMP-7, which is involved in matrix degradation within the atherosclerotic lesion, and is associated with severe atherosclerosis, plaque destabilization, and higher incidence of coronary and cerebrovascular events.

General remarks

Our study confirms that OH and, consequently, cardiovascular autonomic dysfunction are associated with multifactorial mechanisms facilitating cardiovascular disease, including inflammation and autoimmune mechanisms. Elevated levels of midkine, immunoglobulin-like transcript 3, and regenerating islet-derived protein 4 are in accordance with previous studies that have demonstrated association of OH with neurodegenerative and autoimmune diseases[2, 4, 32]. Thus, our findings expand the evidence that OH is not merely a haemodynamic phenomenon, but in fact includes a range of dysregulated molecular events heralding malfunction of the immune and circulatory system.

Strengths and limitations

The present study is based on a large sample of symptomatic individuals and a novel state-of-the-art proteomics chip was used. Furthermore, all patients were examined according to a standardized protocol with beat-to-beat haemodynamic monitoring, thus minimizing the risk of inaccurate or missed diagnosis of OH. Moreover, we performed a sequential two-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. Nevertheless, this may have resulted in omission of significant information, therefore further studies on independent patient samples are necessary.

Some limitations should be also addressed. The study was performed on symptomatic individuals who were unaware of the nature of underlying disorder prior to investigation. Consequently, our study may not be entirely representative of OH

detected in the general population through screening programmes or in asymptomatic outpatients.

Conclusions

Our study confirms and extends the concept that broad-range proteomics analysis can considerably improve the understanding of autonomic failure. We report here that presence of orthostatic hypotension in patients with a history of unexplained syncope and orthostatic intolerance is associated with elevated plasma levels of midkine, immunoglobulin-like transcript 3, and regenerating islet-derived protein 4. These observations support the hypothesis that autonomic dysfunction may be evoked by inflammatory processes, but that it may also maintain a systemic inflammatory milieu with possible detrimental effects on the cardiovascular system.

Contributors: AF, FR, NA, MJ had full access to all the data in the study and take responsibility of the data and accuracy of the data analysis. MJ, OM, RS, AF contributed to the study concept and design. AF, OM, MJ contributed to the acquisition of data. All authors analysed and interpreted the data. AF was the study supervisor. NA, AF, FR did the statistical analysis. MJ, FR, RS, AF drafted the manuscript with critical revision for important intellectual content from all authors.

Transparency: the lead authors (the manuscript's guarantors) affirm that the manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

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Competing interests: All authors have completed the ICMJE uniform disclosure at www.icmje.org/coi_disclosure.pdf and declare: AF reports personal fees from Cardiome Corp. and a patent Thermofisher pending outside the submitted work; RS reports personal fees and other from Medtronic Inc., St. Jude Medical Inc. outside the submitted work; RS performs consultancy for Medtronic Inc.; RS is a member of the speaker's Bureau St. Jude Medical/Abbott Inc.; RS is shareholder in Boston Scientific

Inc., Edwards Lifesciences Inc., Shire PLC, Roche SA and Astrazeneca PLC; no other relationships or activities that could appear to have influenced the submitted work.

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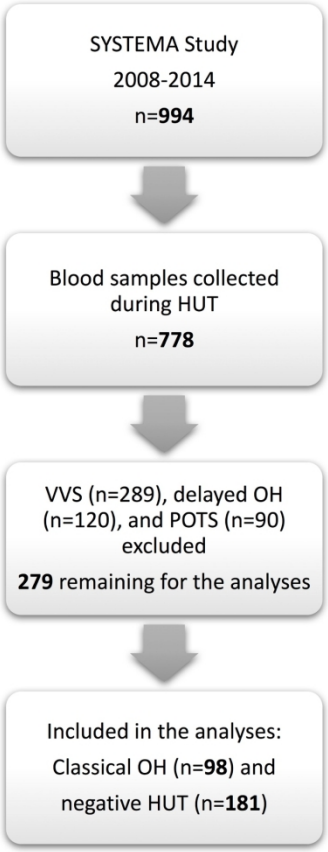
Figure 1. Flow-chart summarising the selection process of study population.



HUT, head-up tilt; OH, orthostatic hypotension; POTS, postural orthostatic tachycardia syndrome; SYSTEMA, Syncope Study of Unselected Population in Malmö; VVS, vasovagal syncope.

Table 1

Patient characteristics according to orthostatic hypotension status (n=279).

Characteristic	OH positive n=98	OH negative n=181	P value
Age (years)	68.1±13.54	59.7±20.5	<0.001
Sex (%, male)	58.2	41.4	0.008
Body-mass index (kg/m^2)	25.2±4.28	25.8±4.70	0.29
Systolic BP supine (mmHg)	137.5±25.6	135.5±20.3	0.47
Diastolic BP supine (mmHg)	72.7±10.2	71.9±9.50	0.54
Heart rate (bpm) supine	69.2±11.9	69.6±11.7	0.78
Systolic BP (mmHg) HUT	85.1±23.1	124.8±19.2	<0.001
Diastolic BP (mmHg) HUT	55.2±13.6	72.5±10.8	<0.001
Heart rate (bpm) HUT max	80.5±14.7	78.1±14.3	0.20
V-pattern at VM (%)	27.3	1.7	<0.001
BRS index (ms/mmHg)	2.9±1	12.3±4.8	0.004
Hypertension (n, %)	50.5	39.8	0.09
Ischemic heart disease (%)	10.2	10.5	0.94
Heart failure (%)	4.1	7.2	0.30
Atrial fibrillation (%)	7.1	8.3	0.73
Diabetes mellitus (%)	5.1	8.9	0.25
Parkinson disease (%)	4	0	0.007
Cancer (%)	14.3	9.9	0.28
Smoking (%)	8.2	19.9	0.04
GFR	70±20	81±24	<0.001
LVEF (%)	54±3	54±3	0.92

Beta-blocker (%)	26.3	31.5	0.36
Diuretic (%)	12.1	11.2	0.82
CCB (%)	15.2	12.4	0.51
ACE-I(%)	19.2	8.4	0.009
ARB (%)	16.2	14.6	0.73
Alpha-blocker	4	1.1	0.11
Long-acting nitrate (%)	1	7.3	0.02

OH, orthostatic hypotension; *P* values for differences between the groups shown as mean and standard deviation for continuous variables and as percentages for categorical variables. ACE-I, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; BP, blood pressure; BRS, baroreflex slope index; CCB, calcium channel blockers; GFR, glomerular filtration rate (MDRD formula); HUT min/max, lowest/highest value during passive head-up tilt test; bpm, beats per minute; LVEF, lef ventricular ejection fraction; VM, Valsalva maneuver.

Table 2

Multiplex proteomics analysis of 4 of 49 oncological biomarkers, selected by supervised multivariate principal component analysis, in 89 patients with classical orthostatic hypotension. Plasma concentrations of the assessed proteins are expressed on a log2-scale. Inter-group differences were assessed using analysis of variance method. Bonferroni-corrected significant values (p<0.0124) are marked in bold.

Biomarker	OH positive (n=98)	OH negative (n=181)	P-value
Immunoglobulin-like transcript 3 (ILT-3)	2.46±0.64	2.18±0.61	**<0.001**
Midkine (MK)	7.30±0.57	7.00±0.59	**<0.001**
Regenerating islet-derived protein 4 (REG-4)	3.68±0.58	3.41±0.57	**<0.001**
Tartrate-resistant acid phosphatase type 5 (TR-AP)	5.36±0.50	5.19±0.52	**0.007**

Table 3

Association between changes in systolic blood pressure during HUT and proteomic biomarkers in univariate and multivariate regression*.

Biomarker	β	95% CI	P-value	β	95% CI	P-value
Immunoglobulin-like transcript 3 (ILT-3)	10.7	5.9 - 15.5	**< 0.001**	7.8	2.4 - 13.3	**0.021**
Midkine (MK)	13.3	8.3 - 18.4	**< 0.001**	10.4	4.9 - 15.9	**0.001**
Regenerating islet-derived protein 4 (REG-4)	12.2	7.1 - 17.41	**< 0.001**	9.6	4.1 - 15.0	**0.003**
Tartrate-resistant acid phosphatase type 5 (TR-AP)	12.2	6.3 - 18.1	**< 0.001**	5.3	-0.6 - 11.2	0.315

*Adjusted for age, sex, supine systolic blood pressure, diabetes mellitus, hypertension, antihypertensive treatment, glomerular filtration rate, presence of cardiovascular disease and smoking.

Author Agreement Form – International Journal of Cardiology

Manuscript Title: Proteomic signature of inflammation in classical orthostatic hypotension

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This statement is to certify that all authors have seen and approved the manuscript being submitted, have contributed significantly to the work, attest to the validity and legitimacy of the data and its interpretation, and agree to its submission to the *International Journal of Cardiology*.

We attest that the article is the Authors' original work, has not received prior publication and is not under consideration for publication elsewhere. We adhere to the statement of ethical publishing as appears in the International of Cardiology (citable as: Shewan LG, Rosano GMC, Henein MY, Coats AJS. A statement on ethical standards in publishing scientific articles in the International Journal of Cardiology family of journals. Int. J. Cardiol. 170 (2014) 253-254 DOI:10.1016/j.ijcard.2013.11).

On behalf of all Co-Authors, the corresponding Author shall bear full responsibility for the submission. Any changes to the list of authors, including changes in order, additions or removals will require the submission of a new author agreement form approved and signed by all the original and added submitting authors.

All authors are requested to disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work. If there are no conflicts of interest, the COI should read: “The authors report no relationships that could be construed as a conflict of interest”.

Figure S1. Quantile regression analyses. 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. The 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.

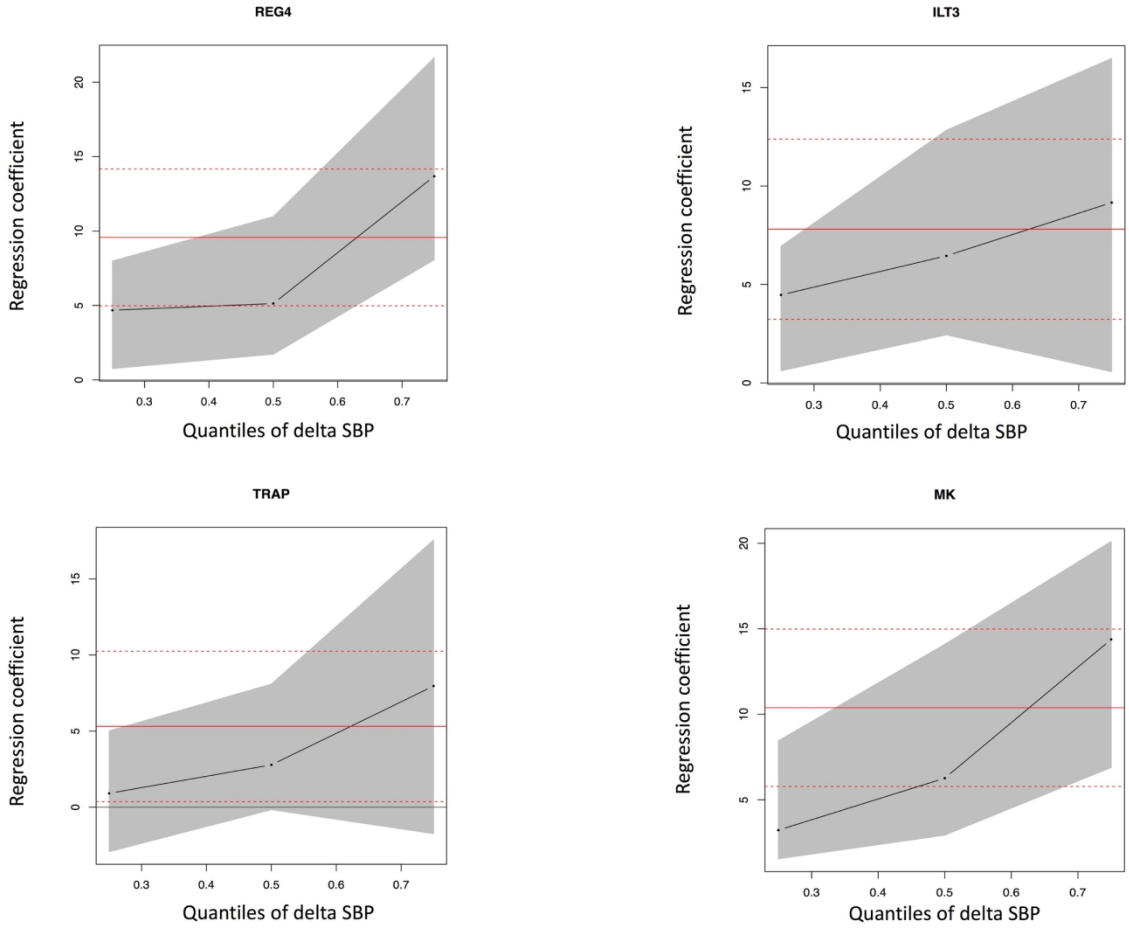

Table S1. Immuno-oncology panel: biomarker list

Amphiregulin (AR)
B-cell activating factor (BAFF)
Cadherin-3 (CDH3)
Carbonic anhydrase IX (CAIX)
Carcinoembryonic antigen (CEA)
Caspase-3 (CAPS-3)
C-C motif chemokine 19 (CCL19)
C-X-C motif chemokine 10 (CXCL10)
C-X-C motif chemokine 11 (CXCL11)
C-X-C motif chemokine 13 (CXCL13)
C-X-C motif chemokine 5 (CXCL5)
C-X-C motif chemokine 9 (CXCL9)
Cyclin-dependant kinase inhibitor 1 (CDKN1A)
Early activation antigen CD69 (CD69)
Epidermal growth factor receptor (EGFR)
Epididymal secretory protein E4 (HE4)
Epithelial cell adhesion molecule (Ep-CAM)
Erythropoietin (EPO)
Eukaryotic translation initiation factor 4B (eIF-4B)
Extracellular matrix metalloproteinase inducer (EMMPRIN)
Ezrin (EZR)
Fas antigen ligand (FasL)
FAS-associated death domain protein (FADD)
Fms-related tyrosine kinase 3 ligand (Flt3L)
Folate receptor alpha (FR-alpha)
Furin (FUR)
ICOS ligand (ICOSLG)
Immunoglobulin-like transcript 3 (ILT-3)
Integrin alpha-1 (ITGA1)
Interferon gamma (IFN-gamma)
Interleukin-2 (IL-2)
Interleukin-12 (IL-12)
Interleukin-17 receptor B (IL-17RB)
Interleukin-7 (IL-7)
Latency-associated peptide transforming growth factor beta-1 (LAP TGF-beta 1)
Lipopolysaccharide-induced tumor necrosis factor-alpha factor (LITAF)
Melanoma-derived growth regulatory protein (MIA)
MHC class I polypeptide-related sequence A (MIC-A)
Midkine (MK)
Myeloid differentiation primary response protein MyD88 (MYD88)
NT-3 growth factor receptor (NTRK3)
Parkinson disease protein 7 (PARK7)
Prostasin (PRSS8)

Receptor tyrosine-protein kinase erbB-2 (ErbB2/HER2)
Receptor tyrosine-protein kinase erbB-3 (ErbB3/HER3)
Receptor tyrosine-protein kinase erbB-4 (ErbB4/HER4)
Regenerating islet-derived protein 4 (REG-4)
Tartrate-resistant acid phosphatase type 5 (TR-AP)
Thrombopoietin (THPO)
Transforming growth factor alpha (TGF-alpha)
Tumor necrosis factor (TNF)
Tumor necrosis factor receptor superfamily member 4 (TNFRSF4)
Tyrosine-protein kinase Lyn (LYN)
Tyrosine-protein phosphatase non-receptor type 22 (PTPN22)
Vascular endothelial growth factor receptor 2 (VEGFR-2)
Vascular endothelial statin (VE-statin)
Vimentin (VIM)