Published in final edited form as:

Proteomics Clin Appl. 2012 December ; 6(0): 615–625. doi:10.1002/prca.201200049.

Autoimmunoreactive IgGs from patients with postural orthostatic tachycardia syndrome

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Abstract

Purpose—Autoantibodies are implicated in the pathogenesis of cardiovascular diseases and cardiac arrhythmias. In this pilot study, we tested the hypothesis that autoantibodies are present in patients with postural orthostatic tachycardia syndrome (POTS).

Experimental design—Seven control subjects (6 F:1 M, average age 36.1 years) and 10 patients with the diagnosis of POTS (7 F: 3 M, average age 35.1 years) provided informed consent and 30 ml of venous blood. Human heart membrane proteins were resolved by 2DE and immunoblotted against purified IgGs from controls and patients.

Results—Eighteen protein spots immunoreactive specifically against patient IgGs were detected and they were excised from gels, trypsin-digested, and analyzed by nanoLC-electrospray tandem MS. Forty unique proteins were identified and these include proteins that are associated with cardiac hypertrophy (mimcan, myozenin), cardiac remodeling (periostin), cardiomyopathy (desmin, desmoplakin), cell survival (laminin), structural integrity (filamin), chaperone proteins (crystalline, HSP70), mitochondrial enzymes and channel proteins. Ingenuity Pathway Analysis showed multiple pathways were involved including those that regulate energy metabolism, redox, fibrosis, cardiac hypertrophy and degeneration.

Conclusions and clinical relevance—Autoantibodies are present in patients with POTS. These autoantibodies cross-react with a wide range of cardiac proteins and may induce alterations in cardiac function. Autoimmune pathogenetic mechanisms should be further explored in these patients.

Keywords

Autoantibodies; cell membrane; proteomics; postural orthostatic tachycardia syndrome (POTS)

1 Introduction

Autoimmunity is an important mechanism that underlies many important disease entities including type 1 diabetes mellitus, systemic lupus erythematosus, Graves' disease,

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The authors have declared no conflict of interest.

myasthenia gravis, celiac disease, arthritis, hemolytic anemia, and hepatitis [1]. Autoimmune diseases are estimated to affect more than 5% of the US population with a striking predilection for females [2]. Autoimmune mechanisms have not been known to play a major role in the pathogenesis of cardiovascular diseases, but there is emerging evidence that autoantibodies are involved in certain cardiovascular disorders, which include dilated cardiomyopathy, myocarditis, vasculitis, congenital complete heart block, and cardiac arrhythmias [3, 4]. Postural orthostatic tachycardia syndrome (POTS) is a disorder of unknown etiology but is an important cause of orthostatic intolerance resulting from cardiovascular autonomic dysfunction [5]. Individuals affected by POTS are mainly young, predominantly female, and has overlapping clinical manifestations with inappropriate sinus tachycardia and autonomic dysfunction and may share common pathophysiological mechanisms [5, 6]. In this study we tested the hypothesis that autoimmunoreactive IgGs are present in patients with POTS, and that these autoantibodies may react with critical cardiac proteins. The goal of this pilot study is to explore new paradigms of disease mechanisms in POTS. We tried to reduce sample complexity by cellular fractionation and analysis is most efficient when combined with 2DE and mass spectrometry (MS) [7]. In this study, we resolved proteins from human cardiac membrane fractions by 2DE, followed by immunoblotting against IgGs isolated from POTS patients. Patient-specific immunoreactive membrane protein spots were identified when compared with immunoblots against healthy control IgGs. The patient-specific protein spots were digested with trypsin followed by proteomic analysis with tandem MS/MS. The results showed autoimmunoreactive IgGs were present in patients with POTS.

2 Materials and methods

2.1 Subjects and samples

This prospective study was approved by the Mayo Clinic Institutional Review Board and informed consent was obtained from each study subject. Patients with the diagnosis of POTS were recruited from the Autonomic Disorders Program of Department of Neurology or from the Heart Rhythm Clinic of Division of Cardiovascular Diseases, Mayo Clinic. All patients fulfilled criteria for POTS. These included heart rate increment >30 bpm, associated with symptoms of orthostatic intolerance and absence of exclusionary criteria [8]. All patients had undergone full autonomic function tests including evaluation of cardiovascular, sudomotor and adrenergic function in the Mayo Autonomic Laboratory. Patients were excluded if they had a history of confirmed autoimmune diseases, or unable to provide consent form or when we were unable to obtain blood samples. Thirty cc of venous bloods were obtained from seven healthy volunteers and ten patients with the diagnosis of POTS. The detailed clinical and laboratory profiles of the subjects and controls are outlined in Table 1.

The blood collected was immediately processed for serum isolation and stored at -80°C until further processing. IgGs were isolated using Melon gel IgG isolation kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instruction.

2.2 Isolation of membrane fractions from human heart tissue

Human heart from a healthy individual was obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA, USA).

Membrane proteins were prepared using ProteoPrep membrane extraction kit (Sigma) according to the manufacturer's instruction. Briefly, the heart tissues from 4 different chambers were pooled, homogenized and sonicated with chilled lysis buffer containing protease inhibitors. The pellet membranes and membrane proteins obtained from

ultracentrifuge (115,000 $\times g$ for 1 hour at 4 °C) were resuspended in Protein Extraction Reagent Type 4 and sonicated on ice. Cell debris was eliminated by centrifugation of the suspension (14,000 $\times g$ for 45 minutes at 15 °C). The supernatant was then reduced by freshly prepared tributylphosphine for 1 hour at room temperature. Membrane proteins were alkylated with iodoacetamide (IAA) for 1.5 h at room temperature with a concentration of 1.9 mg/ml.

2.3 2DE Immunoblot analysis

First-dimension IEF was carried out using the Protean IEF Cell (BioRad). For each sample, 100 μ g of the protein was diluted in 2DE rehydration buffer [7M urea, 2M thiourea, 4% CHAPS, 30mM dithiothreitol (DTT), 0.1% 3–10 Pharmalyte (GE) and 0.25% pH 3–10NL (non-linear) IPG buffer (GE)] and overlaid with 11 cm 3–10NL IPG strip. The strips were passively rehydrated overnight for 11 h, then focused for a total of 25KVhr. IPG strips were stored at –80°C prior to separation by SDS-PAGE. IPG strips were equilibrated in SDS equilibration buffer (6 M urea, 2% SDS, 50 mM Tris pH 8.8, 30% glycerol) for 10 min with 1% DTT, then 15 min in fresh buffer with 2% IAA, to maximize alkylation of any free cysteines. 10.5–14% Criterion gradient gels (133 $\times$ 87 $\times$ 1.0 mm) were used for the second dimension separation. After electrophoresis, gels for spot excision and MS analysis were fixed and silver-stained. Gels for immunoblot analysis were transferred to PVDF (HyBond, GE) using CAPS buffer with 10% methanol. Some transferred blots were also stained with Sypro-Ruby fluorescent blot stain (Invitrogen) for identifying spot location by comparing with immunoblot results. Serum IgG was used for immunoblotting at 0.2 mg/ml. IgGs were diluted with blocking buffer (PBS containing 0.05% Tween 20, 10% milk). Immunoblot analysis was performed as we have previously described [9]. Antibody binding was detected with a horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Invitrogen) and chemiluminescence using SuperSignal West Pico Chemiluminescence Substrate (Pierce Biotechnology). Specific antibodies against desmin (1:1000, Cell Signaling Technology, Inc), and periostin (1:1000, Sigma, St. Louis) were used for 2DE immunoblot analysis to identify their spot locations in 2DE gels.

2.4 Protein identification and data analysis

Immunoreactive protein spots were selected by comparing immunoblot with a silver-stained 2-DE gel. Each spot was given a standard spot number and by its position on a grid of (x by y). The spots were selected visually and by analysis of overlaid images of the immunoblot and the fluorescent stained protein spots on the membrane using PDQuest 7.4.0. image analysis software (Bio-Rad). Protein spots specifically reacting with patient IgGs were selected and processed for protein identification by in-gel trypsin digestion and nano-LC-ESI-tandem mass spectrometry using anLTQ Orbitrap (ThermoElectron, Bremen, Germany) coupled to a nano-LC-2D HPLC system (Eksigent, Dublin, CA). The mass spectrometer experiment was set to perform an Orbitrap full scan from 375 to 1600 m/z, (60,000 resolution at 400 m/z), followed by linear ion trap MS/MS scans on the top five [M + 2H]²⁺ or [M + 3H]³⁺ ions. All MS/MS spectra were analyzed by using Mascot (version 2.2.04; Matrix Science, London, UK;), Sequest (ThermoFinnigan, San Jose, CA; version 27, rev. 12), and X! Tandem (www.thegpm.org; version 2006.09.15.3) Each analysis was set up to search the most current Swiss-Prot database, assuming semitrypsin or full trypsin digestion with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 10.0 PPM (Swiss-Prot is provided in the public domain by the Swiss Institute of Bioinformatics, Geneva, Switzerland, <http://www.expasy.ch/sprot/>). Oxidation of methionine and iodoacetamide derivatives of cysteine were specified as variable modifications. Scaffold proteomics software (Scaffold, ver. 2.00.02; Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at a higher than 95.0% probability, as specified

by the Peptide Prophet algorithm [10]. Protein identifications were accepted if they could be established at a higher than 95% probability and contain at least two unique peptides. Protein probabilities were assigned by the Protein Prophet algorithm [11]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

2.5 Protein interaction network analysis

An industry package, Ingenuity Pathway Analysis (IPA, Ingenuity® Systems, Redwood City, CA) for pathway and network analysis was used to identify the signaling pathways and networks associated with the autoantibody-targeted proteins. Briefly, identified proteins from spots that specifically reacted with patient IgGs were submitted as focus proteins for network analysis to identify associated functional networks. A composite interactome showing an overview of protein to protein interactions was constructed by merging subnetworks reported by IPA. The composite was laid out graphically using the network visualization algorithm software Cytoscape 2.8.2 (<http://www.cytoscape.org>) [12], with network topology assessed as an undirected network using Network Analyzer [13, 14]. Network architecture was defined by degree (k), the number of node connections, and node degree distribution ($P[k]$), the probability that a node has k links, where $P[k] = X[k]/n$, when $X[k]$ is the number of nodes with degree k and n is the total number of network nodes [15, 16]. $P[k]$ versus k discriminates between random and scale-free topographies, defined by normal and power law distributions, respectively [14]. The Anderson-Darling normality test [15, 17] was used to rule out a normal distribution, so $P[k]$ versus k was calculated as a power law relationship using a cumulative distribution function [16] to determine in the power law distribution ($P[k] \sim k^{-\gamma}$), using eq 1:

$$\gamma = 1 + n \left[\sum_{i=1}^n \ln \frac{x_i}{x_{\min}} \right]^{-1} \quad (1)$$

where γ is the power law exponent, n is the number of network nodes, x_i is the node degree, and $x_{\min}$ is the minimum node degree within the network, with statistical error [16] for eq 1 defined by eq 2:

$$\sigma = \sqrt{n} \left[\sum_{i=1}^n \ln \frac{x_i}{x_{\min}} \right]^{-1} = \frac{\gamma - 1}{\sqrt{n}} \quad (2)$$

]

3 Results

3.1 2DE gel immunoblotting, protein spot matching and protein identification

To identify the potential cardiomyocyte membrane protein targets of autoantibodies in patients with POTS, we used the membrane preparation of a human heart from a healthy donor. Following 2DE separation and silver staining, 801 protein spots were visualized using PDQuest (Bio-Rad Laboratories, Inc.). A representative image of a silver stained 2DE gel is shown in Figure 1A. Analysis of the 2DE gel immunoblots showed that serum IgGs from healthy controls and POTS patients reacted positively with some of the heart membrane spots but with different patterns; examples are shown in Figure 1B. Identification of immunoreactive protein spots was aided by merging images of Sypro Ruby stained PVDF membrane (pseudo-color in red) with that of an immunoblot (pseudo-color in green). The yellow spots suggest protein spots immunoreactive against patient IgG (Figure 2, upper panel). Based on the immunoblots from 7 controls and 10 patients, 18 patient-specific

immunoreactive protein spots were identified and sampled for further analysis by MS/MS and proteomics (Figure 2B, Table 2 and supplement Table 1). Sixteen spots reacted with both healthy controls and patients (Figure 2B and Table 3).

Protein identification of these patient-specific protein spots by nanoLC-electrospray tandem MS identified 40 unique proteins (Table 4). The list of putative immunoreactive proteins against the IgGs of patients with POTS can be broadly categorized into those involved with transcriptional regulation, chaperone functions, signaling mechanisms, cytoskeletal structure, cellular metabolism, and redox regulation (Table 4). Specific proteins may include those that are associated with cardiac hypertrophy (mimecan, myozenin), cardiac remodeling (periostin), cardiomyopathy (desmin, desmoplakin), cell survival (laminin), structural integrity (filamin), chaperone proteins (crystalline, HSP70), mitochondrial enzymes (NADH dehydrogenase) and channel proteins (voltage-dependent anion-selective channels).

To validate the results of nanoLC-MS/MS, we used commercially available antibodies specifically against periostin and desmin for 2DE immunoblot analysis on the same human heart membrane preparation. Desmin was identified in 2DE protein spot # 12 (Figure 2) through nanoLC-MS/MS. Matching spot in 2DE immunoblot with that of #12 in the gel reacted positively with the anti-desmin antibody. Periostin was identified at spot #2 in 2DE gel (Figure 2B), where anti-periostin antibody reacted positively in the 2DE immunoblot (Figure 3). Anti-periostin antibody also reacted with dots that close to spot #2. However it could not decide if those spots contained periostin since we had not sampled these spots for ID analysis. In general, the results from 2DE immunoblotting using specific antibodies are in agreement with those obtained from patient serum IgG and the proteomic analysis, hence, validating the presence of specific proteins in spots identified by mass spectrometry-proteomic analysis.

3.2 Network and pathway analysis

To gain more insights into the physiological and pathophysiological relevance of the autoimmune-targeted proteins, we performed network and canonical pathway analysis using Ingenuity Pathway Analysis to organize our results. Proteins from Table 4 were integrated into a composite neighborhood comprised of 70 nodes linked by 109 interactions or edges (Figure 4). Network topology was assessed as an undirected network and the results demonstrated that the network topology was not random. A nonstochastic property was confirmed by examination of the interrelationship between node degree (k) and degree distribution ($P[k]$) (Figure 4). The organized assemblage followed a power law distribution indicative of scale-free architecture, where $P(k) \sim k^{-\gamma}$, with $\gamma = 1.64 \pm 0.31$ falling within the predicted confidence range of biological networks [14, 15].

Canonical pathway analysis demonstrated that many of the proteins targeted by POTS patient serum IgGs are interrelated in pathways based on the IPA library (Table 5). Many pathways were involved including those that regulate energy metabolism, redox, fatty acid metabolism, fibrosis, cardiac hypertrophy and degeneration (Table 5).

4 Discussion

A key finding of this study is that immunoreactive IgGs against human cardiac proteins are present in patients with POTS. These autoantibodies were found to react against a wide range of cardiac membrane proteins that regulate various cardiac functions. To our knowledge, circulating autoantibody from POTS patient targeting cardiac proteins has not been reported previously. How these autoantibodies may affect in the clinical manifestation of POTS is currently unknown, but the presence of autoantibodies may predispose the heart

to vulnerable pathologic stimuli, and an adverse autoimmune reaction may trigger possible inflammatory responses with injury to the myocardium.

The exact triggers for induction of autoimmune responses are not well known, cardiac autoimmunity can be triggered by autoantigens presented to the immune system following cardiac injury induced by endogenous or exogenous factors (such as viral infections). Molecular mimicry and cross-reactivity may play an important role in inducing an autoimmune response, especially in individuals with Chagas disease [18]. POTS is characterized by orthostatic intolerance with an excessive heart rate increment upon assuming an upright position and onset of the syndrome has been linked to infection, trauma, surgery or stress [5]. However, the mechanism that underlies the tachycardic response in POTS patients is poorly understood. Most of the patients are young (aged between 15 years and 40 years) and the incidence is higher in women [19], who are also more often affected by autoimmune diseases than men [2]. Previous study showed that anti-ganglionic acetylcholine receptor (AChR) antibodies are present in 10-15% of patients with POTS [8, 20, 21], suggesting that autoimmune mechanisms are important in the pathogenesis of POTS. AChR was not one of the cardiac membrane proteins detected in our study as the AChR in hearts are of the muscarinic and not the neuronal nicotinic subtype. However, autoantigen profiling may provide insights into its pathogenesis, and may facilitate development of novel approaches for the diagnosis and therapy for these patients.

Current technologies for proteomic studies provide efficient tools in searching antigens and antibodies in autoimmune diseases [22, 23]. The nano-ESI-LC/MS/MS allow comprehensive identification of proteins targeted by autoimmune antibodies by determining a high coverage of peptide sequences. Although 2DE is considered to be one of the best methods for separating complex protein mixtures [22], and the analytical power of MS-based technologies has greatly improved in recent years, sample preparation for proteomic analysis is still a central part for maximizing the sensitivity of protein identification. In extremely complex protein mixtures, high-abundance protein in a sample may limit both loading detectable amount of low-abundance proteins in 2DE and detecting sensitivity for MS, as a result, reducing the sensitivity of detecting low abundant proteins. To reduce sample complexity we chose to prepare cardiac membrane proteins to enhance the sensitivity for detecting membrane related protein targets. The results of our study showed that the membrane associated proteins were significantly enriched and many of the putative autoantigens have not been previously suspected to be targets in autoimmunity.

Forty unique proteins were identified as putative targets of autoantibodies in POTS (Table 4). Many of the proteins have previously been implicated in cardiac dysfunction or cardiac disease, such as cardiac hypertrophy (mimcan, myozenin) [24, 25], cardiac remodeling (periostin) [26], cardiomyopathy (desmin, desmoplakin) [27–29], cell survival (laminin) [30], structural integrity (filamin) [31], chaperone proteins (crystalline, HSP70) [32], mitochondrial enzymes and channel proteins. These results suggest that immune interaction with a wide range of cardiac proteins may contribute to the cardiovascular dysfunction in POTS and demonstrated feasibility using this type of approach in the study of clinical syndromes. These results may serve as a launching pad for further studies on the autoimmune mechanisms associated with POTS and for determination of the functional role of the target proteins on the clinical manifestation of POTS.

Desmin and periostin were chosen for validation of the nanoLC-MS/MS results because high quality affinity purified antibodies were available commercially for 2DE immunoblot analysis of human heart membrane proteins (Figure 3). The results confirmed that the immunoreactive protein spots are in agreement with those from mass spectrometric-proteomic analyses indicating that this technology is a valuable approach in identifying

potential autoimmune targets. Desmin and periostin have been shown to associate with cardiovascular diseases [26, 29]. Desmin is a class-III intermediate filaments found in muscle cell and defects in desmin has been shown to cause myopathy, cardiac conduction blocks, arrhythmias, restrictive heart failure, sudden cardiac death [28]. In addition, study also showed that mice without desmin were weaker, fatigued more easily, and had impaired mitochondrial function [33]. Currently, the functional consequences of autoimmune reaction to desmin are unknown and deserve further investigation. Periostin is a secreted extracellular matrix protein associated with areas of fibrosis and is a potent inducible regulator of cellular reorganization and extracellular matrix homeostasis, playing an important role in cardiac remodeling [26]. Recent studies suggested that periostin is important in cardiac regeneration after myocardial infarction by inducing proliferation of differentiated cardiomyocytes and promoting cardiac repair [34]. The role of periostin in association with POTS deserves further examination.

In conclusion, auto-immunoreactive IgGs are present in patients with POTS. The autoantibodies react against a wide range of cardiac membrane proteins including those not previously reported to be autoimmune targets or known to be associated with POTS. We believe the methods we have adopted in this study are useful in helping identify potential targets of autoantibodies. Further studies are needed to establish the functional role of autoantigen-autoantibody interaction in these patients.

5 Limitations of the study

This is a pilot study involving a small number of patients with POTS and controls. Detection of autoantibodies could be limited by focusing on specific cellular fractional cardiac membrane proteins. We have identified a list of 40 unique cardiac membrane proteins that are potential targets of autoantibodies in patients with POTS. However, further confirmation using an independent binding assay to screen a much larger number of patients and controls is needed. In addition, the functional consequence of IgG binding to specific target cardiac membrane proteins is unknown and will require functional assays using IgGs from controls and patients on cardiomyocytes in culture. These studies are beyond the scope of the current study but will be needed to further advance our understanding on the autoimmune mechanisms that may underlie the pathogenesis of POTS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Dr. Kent Arrell (Mayo Clinic, Rochester) for his assistance on network analysis, and Mayo Proteomics Core for protein identification.

This work was supported by grants from the National Institutes of Health HL74180 and HL080118 (to Hon-Chi Lee), and the Mayo Clinic Foundation (to Win-Kuang Shen).

Abbreviations

POTS	postural orthostatic tachycardia syndrome
IPA	Ingenuity Pathway Analysis

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Statement of clinical relevance

Autoimmunity is an important mechanism that underlies many important diseases and autoimmune diseases are estimated to affect more than 5% of the US population with a striking predilection for females. There is emerging evidence that autoantibodies are involved in cardiovascular disorders including certain cardiomyopathies and cardiac arrhythmias. Recently, autoantibodies were implicated in the pathogenesis of autonomic dysfunction and inappropriate sinus tachycardia. Postural Orthostatic Tachycardia Syndrome (POTS) is a disorder of unknown etiology but has overlapping clinical manifestations with inappropriate sinus tachycardia and may share common pathophysiological mechanisms. The goal of this project is to explore autoimmunity as a new paradigm for mechanisms of cardiovascular diseases using proteomic and mass spectrometric approaches. This study demonstrated that autoantibodies are present in patients with POTS and autoantibodies should be further explored as novel biomarkers in these patients.

Figure 1. Proteome and 2DE Western blotting of human heart membrane fractions
(A) The proteome revealed by 2DE at NL pH 3–10 isoelectric focusing, SDS-PAGE and silver staining. (B) Protein spots in the membrane fractions reacted with IgGs purified from healthy (upper panel) and patient (lower panel) serum as revealed by 2DE immunoblotting. Arrow shows specific immunoreactions in patient spot.

A **B** **Figure 2. Identification of Immunoreactive Protein Spots**

(A) PVDF membrane was stained with SyproRuby (pseudo-color in green) and 2DE gel with silver stain (pseudo-color in red). The two images were overlapped using PD-Quest to facilitate identification of antigenic proteins (yellow). (B) Patient-specific immunoreactive spots sampled from silver stained 2DE gel for protein identification, spots 1 to 18; spots that reacted with both control and POTS patient IgGs, spots 19 to 34.

Figure 3. 2DE immunoblotting analyses of desmin and periostin on human heart membrane protein

(A) Immunoblot analysis of desmin (left panel). The result shows that the specific immuno reactive spot is in agreement with those obtained from patient serum IgG (right panel). (B) Immunoblot analysis of periostin (left panel). The result shows that the immuno reactive spot overlaps with the spot from patient serum IgG (right panel).

Figure 4. Network analysis of the proteins identified by MS from spots reacted with patient serum IgGs

(A) 40 identified proteins by MS submitted to Ingenuity Pathways Analysis as focus nodes were integrated into a composite neighborhood comprised of 113 protein interaction network linked by 389 interactions or edges. Nodes are listed by Swiss-Prot gene designations and the color corresponds to ontological function. (B) Network degree distribution, $P(k)$ versus degree (k), followed a power law distribution indicating nonstochastic scale-free network architecture a typical biological networks.

Table 1

Clinical profiles and laboratory findings of controls and POTS patients

Age	Sex	Allergies	PMH	Medications	Symptoms & Study Findings	ECG HR BPM	Holter HR BPM Avg (range)	Tilt Study Baseline HR (BP)	Tilt Study 10 min HR (BP)	ECHO (LVEF %)
Controls										
1	43	F	Codeine, procaine	Benign thyroid nodule	Levapro					
2	23	F								
3	45	F	Lipids, LBP, Herpes zoster							
4	37	F	Azithromycin, Pseudoephedrine	Lipids						
5	44	F	PCN, shellfish	Sinus rhinitis	Advil, fish oil, Imitrex					
6	24	F		Asthma	Alavert					
7	37	M	HBP, GERD		Lisinopril, Prilosec					
POTS Subjects										
1	44	F	Sulfur, Levofloxacin	Melanoma, meningitis, lipids, fibromyalgia, eczema	RetinA cream	126	101 (70–169)	99 (122/80)	122 (110/84)	Normal (60–65%)
2	51	F		Bipolar, HBP	Catapres, Metoprolol, Lipitor, Clonidine, Provera	117	114 (89–165)	92 (148/92)	134 (125/76)	Normal (62%)
3	48	M	Fibromyalgia, chronic fatigue, PTSD		Vit D, Lyrica, Imitrex, Inderal, Florinef	63	73 (50–145)	74 (122/78)	125 (110/70)	Normal (60%)
4	56	M	GI dysmotility		Florinef, Neurontin, K, Midodrine, Mestinon	67		64 (164/94)	64 (50/–) (1.5 min)	
5	35	F	Asthma, lipids, depression		Klonopin, Midodrine, Nadolol, BCP, inhaler	88	93 (64–152)	88 (122/74)	165 (104/82)	Normal (63%)
6	18	F	Hydrocodone	Irritable bowel, hypovitaminosis D	Metoprolol, Citalopram, vit D	88		76 (108/64)	120 (100/80)	

Age	Sex	Allergies	PMH	Medications	Symptoms & Study Findings	ECG HR BPM	Holter HR BPM Avg (range)	Tilt Study Baseline HR (BP)	Tilt Study 10 min HR (BP)	ECHO (LYEF %)
7	20	F	Amoxacillin	Allegra, Microgestin	ortho tolerance, hyperadrenergic state	92		82 (110/56)	131 (100/70)	Normal (61%)
8	21	F	Tramadol, Sprintec	Mononucleosis, irritable bowel	Florinef, Neurontin, Lexapro, Metoprolol, Prilosec, Mestinon	67	90 (64–137)	72 (92/64)	101 (86/64)	
9	26	F	Amoxacillin	Eczema, GERD, sinus, periurethral cyst	Klonopin, Microgestin, Zyrtec, Norco, Cymbalta	82	84 (67–137)	83 (114/70)	137 (108/84)	
10	32	M		HBP, lipids, arthritis, asthma, GI dysmotility	Flexeril, Florinef, Neurontin, Klonopin, Mestinon, Propranolol	71	87 (54–136)	80 (110/76)	131 (112/78)	

Table 2

POTS patient specific immunoreactive spots

Patient #	Patient specific spots*
1	8, 9, 10, 11, 14, 15, 16
2	1, 2, 11, 12, 13
3	1, 2, 17, 18
4	12, 13
5	1, 2, 6, 7, 11
6	3, 4, 5, 14, 15, 16
7	8, 9, 10 11
8	
9	
10	

* Spot code refer to Figure 2B

Table 3

Spots reacted with both healthy control and POTS patients

Control (Ctr); POTS	Spots #*
Ctr 6; POTS 1, 2, 3, 5, 7, 8	19
Ctr 6; POTS 1, 2, 3, 5, 7, 8	20
Ctr 7; POTS 1, 2, 5	21
Ctr 7; POTS 1, 2, 3, 4, 5	22
Ctr 7; POTS 1, 2, 3, 4, 5	23
Ctr 2; POTS 5, 7, 8, 9	24
Ctr 2, 3, 4, 5, 7; POTS 1, 2, 3, 4, 5, 6, 7, 8, 9, 10	25
Ctr 1; POTS 2, 3, 4, 5, 6, 7	26
Ctr 1, 3; POTS 2, 3, 4, 5, 6, 7, 10	27
Ctr 2,3; POTS 1, 2, 5, 6, 7, 9	28
Ctr 2,3; POTS 1, 2, 5, 6, 7, 9, 10	29
Ctr 2; POTS 1, 2, 6, 9, 10	30
Ctr 1, 2; POTS 2, 7, 8, 9	31
Ctr 5; POTS 4	32
Ctr 5; POTS 4	33
Ctr 5; POTS 4	34

* Spot code refer to Figure 2B

Table 4

Unique immunoreactive proteins against IgGs from patients with POTS

Symbols		Protein Name	Function
Transcription	FHL2	Four and a half LIM domains protein 2	Regulation of transcription
Chaperone	CRYAB	Alpha-crystallin B chain	Chaperone, cellular architecture
	HSP71	Heat shock 70 kDa protein 1A	Chaperone, protein folding
	TCPH	T-complex protein 1 subunit eta	Molecular chaperone
Signaling	POSTN	Periostin	Cardiac remodeling
	FGB	Fibrinogen beta chain	Signaling transduction, blood coagulation
	SLMAP	Sarcolemmal membrane-associated protein	Cell cycle
Cytostructure	COL1A1	Collagen alpha-1(I) chain	Fibrosis, tissue structure and integrity
	COL6A3	Collagen alpha-3(VI) chain	Matrix organization, fibrosis
	DES	Desmin	Intermediate filament, CM
	DSP	Desmoplakin	Desmosomal protein, CM
	LAMA2	Laminin subunit alpha-2	Basement membrane, cardiac hypertrophy
	MIME	Mimecan	Matricellular protein, cardiac hypertrophy
	FLNC	Filamin-C	Membrane protein anchor, cytostructure
	MYH7	Myosin-7	Contractile protein, hypertrophic CM
	MYBPC3	Myosin-binding protein C, cardiac-type	Contractile protein, hypertrophic CM
	MYOZ2	Myozenin-2	-actin calcineurin regulation, hypertrophic CM
Metabolism	AL4A1	Delta-1-pyrroline-5-carboxylate dehydrogenase	Amino acid metabolism
	DLD	Dihydrolipoyl dehydrogenase	Acetyl CoA synthesis, glycolysis
	FUMH	Fumarate hydratase	Krebs cycle, amino acid metabolism
	MCCC2	Methylcrotonoyl-CoA carboxylase beta chain	Amino acid metabolism
	CYB5R3	NADH-cytochrome b5 reductase 3	Fatty acid, cholesterol and drug metabolism
	PCCB	Propionyl-CoA carboxylase beta chain	Fatty acid metabolism
	ODPB	Pyruvate dehydrogenase E1 component subunit beta	Krebs cycle, glycolysis
	KPYM	Pyruvate kinase isozyme M1	Glycolysis, anemia
	AOFB	Amine oxidase [flavin-containing] B	Metabolism of bioactive amines
	ECHA	Trifunctional enzyme subunit alpha	Fatty acid metabolism
Oxidoreductase	UQCR1	Cytochrome b-c1 complex subunit 1	Electron transport, ATP
	UQCR2	Cytochrome b-c1 complex subunit 2	Electron transport, ATP
	UCRI	Cytochrome b-c1 complex subunit Rieske	Electron transport, ATP
	COX2	Cytochrome c oxidase subunit 2	Electron transport
	ETFA	Electron transfer flavoprotein subunit alpha	Electron transport, metabolic disorder
	MTCH2	Mitochondrial carrier homolog 2	Mitochondrial function, apoptosis
	IMMT	Mitochondrial inner membrane protein	Mitochondrial structure and function
	NDUFS2	NADH dehydrogenase [ubiquinone] iron-sulfur protein 2	NADH oxidation
	SAMM50	Sorting and assembly machinery component 50 homolog	Mitochondrial structure and transport

Symbols		Protein Name	Function
	COQ6	Ubiquinone biosynthesis monooxygenase COQ6	Oxidoreductase, apoptosis
	VDAC1	Voltage-dependent anion-selective channel protein 1	Oxidoreductase, apoptosis
	VDAC2	Voltage-dependent anion-selective channel protein 2	Oxidoreductase, apoptosis
	VDAC3	Voltage-dependent anion-selective channel protein 3	Oxidoreductase, apoptosis

*
CM: cardiomyopathy

Table 5

Canonical pathways involved in proteins targeted by autoantibodies

Ingenuity Canonical Pathways	Molecules (Symbols)
Valine, Leucine and Isoleucine Degradation	ALDH4A1, PCCB, ALDH6A1, HADHA, MCCC2
Mitochondrial Dysfunction	NDUFS1, UQCRC2, CYB5R3, NDUFS2, COX2, UQCRC1
Oxidative Phosphorylation	NDUFS1, UQCRC2, ATP5H, NDUFS2, COX2, UQCRC1
Propanoate Metabolism	ALDH4A1, PCCB, ALDH6A1, HADHA
-alanine Metabolism	ALDH4A1, ALDH6A1, HADHA
Fatty Acid Biosynthesis	PCCB, MCCC2
Ubiquinone Biosynthesis	NDUFS1, NDUFS2, ALDH6A1
Pyruvate Metabolism	ALDH4A1, DLD, HADHA
Citrate Cycle	ACO2, DLD
Lysine Degradation	ALDH4A1, PRSS3, HADHA
Glycolysis/Gluconeogenesis	ALDH4A1, DLD
Atherosclerosis Signaling	COL1A2, COL1A1
Synthesis and Degradation of Ketone Bodies	HADHA
Fatty Acid Metabolism	ALDH4A1, HADHA
Aryl Hydrocarbon Receptor Signaling	ALDH4A1, ALDH6A1
Tryptophan Metabolism	ALDH4A1, HADHA
Glyoxylate and Dicarboxylate Metabolism	ACO2
Ascorbate and Aldarate Metabolism	ALDH4A1
Acute Phase Response Signaling	FGB, FGG
ILK Signaling	FLNC, DSP
Glutamate Metabolism	ALDH4A1
Alanine and Aspartate Metabolism	DLD
Glucocorticoid Receptor Signaling	COX2, FGG
LXR/RXR Activation	COX2
Xenobiotic Metabolism Signaling	ALDH4A1, ALDH6A1
Caveolar-mediated Endocytosis Signaling	FLNC
Glycine, Serine and Threonine Metabolism	DLD
Arginine and Proline Metabolism	ALDH4A1
TR/RXR Activation	COL6A3
Inositol Metabolism	ALDH6A1
PPAR Signaling	COX2
IL-6 Signaling	COL1A1
Glycerolipid Metabolism	ALDH4A1
Protein Kinase A Signaling	FLNC
Purine Metabolism	ATP5H