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Biomarker discovery by plasma proteomics in familial Brugada Syndrome

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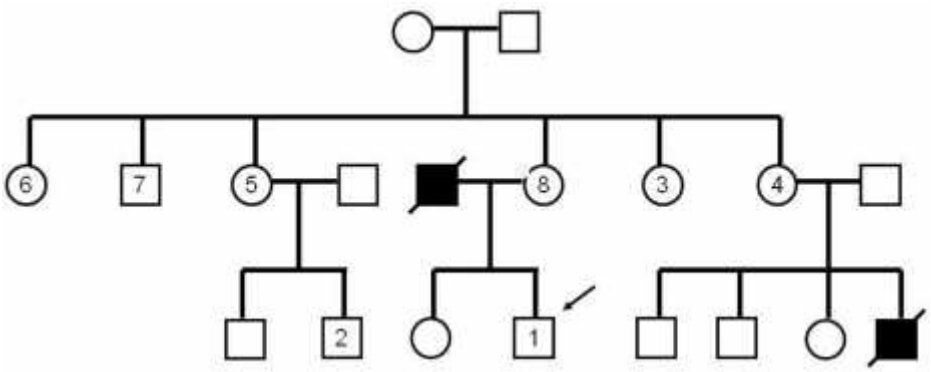
1. ABSTRACT

Brugada Syndrome (BS) is a polygenic inherited cardiac disease characterized by life-threatening arrhythmias and high incidence of sudden death. In this study, two-dimensional gel electrophoresis (2D-PAGE) coupled to mass spectrometry (LC-MS/MS) was used to investigate specific changes in the plasma proteome of BS patients and family members sharing the same gene mutation ($SCN5A^{Q1118X}$), with the aim to identify novel disease biomarkers. Our data demonstrate that the levels of several proteins were significantly altered in BS patients compared with controls. In particular, apolipoprotein E, prothrombin, vitronectin, complement-factor H, vitamin-D-binding protein, voltage-dependent anion-selective channel protein 3 and clusterin were considerably increased in plasma sample of BS patients, whereas alpha-1-antitrypsin, fibrinogen and angiotensinogen were considerably decreased; moreover, post-translational modifications of antithrombin-III were detected in all affected individuals. On the light of these results, we hypothesize that these proteins might be considered as potential markers for the identification of disease status in BS.

2. INTRODUCTION

Brugada syndrome (BS) is a clinically and genetically heterogeneous disease firstly described in 1992 (1), characterized by ventricular arrhythmias, syncope or sudden death in the presence of a specific ECG pattern with a J elevation >=2 mm (0.2 mV) and a coved type ST segment followed by a negative T wave in at least two right precordial ECG leads (so-called type 1 ECG) in patients with structurally normal hearts (1, 2). The diagnosis of BS, mainly based on ECG criteria, is often quite difficult because of the incomplete penetrance of the diseased gene. The incidence of BS ranges from 5 to 66 per 10.000 in Southeast Asia and has a much lower incidence in Europe and the United States (3). Genetically, BS is an autosomal dominant disorder, linked to mutations in genes encoding channel proteins and involved in the generation/propagation of the action potential of the heart. Currently, more than 100 mutations in seven genes have been associated with BS, leading to a classification in which 5 types are described: a) BS type I, due to mutations in the SCN5A gene; b) BS type II, produced by defects occurring in the GPD1L gene; c) BS type III, in which



Pt. N°	INITIALS	MUTATION ($SCN5A^{Q1118X}$)	ECG
1	G.A.	+	+
2	B.A.	+	+
3	B.C.	+	-
4	B.A.	+	-
5	B.M.	-	-
6	G.P.	+	-
7	B.L.	+	-

Figure 1. Genetic tree of the BS family enrolled in the study and presence/absence of typical ECG abnormalities depending on the SCNA5 mutation status. The arrow indicates the proband.

mutations in the CACNA1C gene may occur; d) BS type IV, due to alterations in the CACNB2 gene; e) BS type V, secondary to mutations in the SCN1B gene. Loss-of-function mutations of the SCN5A gene, coding for the depolarizing I (Na) current, are responsible for about 15-30% of BS cases, therefore representing the most common BS genotype (4-7). There are at least four mechanisms by which the SCN5A mutation can influence the function of the sodium channel: 1) failure to express the channel protein; 2) shift in the voltage dependence and time dependence of the sodium channel current activation, inactivation or reactivation; 3) entry of the sodium channel into an intermediate state of inactivation from which it recovers more slowly; 4) accelerated inactivation of the sodium channel (4). Functional studies performed with expression systems, reproducing – both *in vitro* and *in vivo* - some of the above-mentioned gene defects, have demonstrated to cause, for most of the mutations, a loss of function of the sodium channel current (INa) (8). This loss of function is achieved either through a quantitative decrease in the sodium channels due to a failure in their expression or through a qualitative dysfunction of the sodium channels due to impaired kinetics (a shift in the voltage and time dependence activation, inactivation or reactivation; an entry into an intermediate state of inactivation; or an accelerated inactivation).

The lack of reliable tools for the identification of BS in particular subsets of individuals – for example subjects with an abnormal ECG but completely asymptomatic or patients who have been initially diagnosed as suffering syncopal episodes of unknown cause, in whom the ECG changes spontaneously during follow-up from normal to the typical pattern of the syndrome – makes the availability of additional, more sensitive, biomarkers an important issue to be pursued. In fact, even if identification of a recognized mutation will facilitate screening of relatives, in many cases a specific mutation is not found. Such genetic heterogeneity may explain the heterogeneity of expression between individuals.

In this study, we describe a novel proteomics-driven approach for the identification and detection of potential markers of BS through a straight-forward analysis of the plasma that might be useful as additional tool for early diagnosis and follow-up of these high risk individuals.

3. MATERIALS AND METHODS

3.1. Sample collection

Samples were collected at the Cardiology Unit, Magna Graecia University, Medical School, Catanzaro, Italy, and officially registered. All patients and healthy relatives were asked to give informed written consent approved by the ethical committee of the Medical School. Diagnosis of BS was made by two distinct physicians according to clinical and instrumental manifestations; the presence of a heterozygous nonsense S5CNA mutation (Q1118X) was demonstrated in the proband and in 6 relatives by genetic analysis (Figure 1).

Blood plasma was obtained from 16 family members and processed according with HUPO plasma proteome guidelines (Rai AJ *et al.*, 2005). Briefly, 10 mL of blood were drawn by venipuncture and collected in a K_2EDTA tubes. Each sample was subjected to centrifugation (1300 x g for 10 min) within 2 hours of collection and multiple 20 µL aliquots were stored into silicon tubes at -80°C until use. Two distinct pools of plasma from S5CNA^{Q1118X} mutation carriers (pool 1) and healthy, mutation-free, relatives (pool 2) were made.

3.2. ProteoMiner™ enrichment

In order to reduce the degree of plasma complexity, each sample was pre-fractioned using the ProteoMiner™ kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol. This technology is based on treatment of complex biological specimens with a large hexapeptide library bound to a chromatographic support. Each hexapeptide is able to bind, at least in theory, to a unique protein and binding is limited by the bead capacity. Under these conditions, high-abundance proteins rapidly saturate their ligands, so that excess protein is washed out during the procedure; on the other hand, low-abundance proteins are concentrated on their specific hexapeptides, thereby decreasing the dynamic range of proteins in the sample.

3.3. Quantification of plasma protein concentration

Protein concentration was determined before and after ProteoMiner^{TM} treatment according to dye-binding Bradford method (Biorad Laboratories Inc, Hercules, CA) using BSA as a standard.

3.4. Two-dimensional PAGE separation

Plasma pools 1 and 2 were thawed and 140 µg of proteins for each pool was acetone-precipitated by overnight incubation at -20°C. Following centrifugation (10,000xg for 30 minutes), pellets were resuspended in rehydration buffer containing 7M urea, 2 M thiourea, 4% (w/v) CHAPS, 50 mM DTT, 0.8% (v/v) ampholytes (pH 3-10) and applied to 24 cm non-linear IPG strips pH 4-7 (GE Healthcare). First dimension separation was performed at a constant temperature of 16°C with stepwise increase voltage for the time period necessary to reach for a total of 75,000 Vh in total. The IE-focused strips were subsequently incubated for 15 min with equilibration buffer containing 6 M urea, 20% (v/v) glycerol, 2% (w/v) SDS, 0.05 M Tris/HCl pH 8.8 and 2% DTT. Free thiol groups protein alkylation was performed for 15 min in the same solution containing 2.5% (w/v) iodoacetamide instead of DTT. Second dimension was carried out with an Ettan Daltsix Electroforesis System (GE Healthcare) at 20°C, overnight. Strips were loaded on the top of a 10% SDS-polyacrylamide gel and fixed with agarose. Power was held constant to 2 W per gel until the bromophenol blue line reached the bottom of the gel. Three gels were prepared from each sample, fixed for 2 hours in a solution containing 40% ethanol and 10% acetic acid and finally stained with Dodeca^{TM} Silver Stain Kit according to manufacturer's instructions.

3.5. Image analysis and In-gel digestion

Two-dimensional silver-stained gels were scanned using a UMAX Magic Scanner (GE Healthcare) at 1,200 dpi resolution and analyzed by the Image Master 2D Platinum software (GE Healthcare). Selected spots were excised from gels for protein identification and washed with $K_3Fe(CN)_6$ and $Na_2S_2O_3$. Then gel pieces were washed with NH_4HCO_3 at 37 °C. Finally, tryptic digestion was carried out overnight at 37 °C as described elsewhere (9).

3.6. LC-MS/MS analysis

Peptide samples were purified and concentrated using PepClean^{TM} C-18 Spin Columns. Each spin column contains a porous C-18 reversed-phase resin with good binding and recovery characteristics at a wide range of peptide concentrations. PepClean^{TM} C-18 Spin Columns remove interfering contaminants and release peptides in MS-compatible solutions, resulting in increased sensitivity and high-quality spectra.

Peptides were fractionated and fragmented by using a high-performance hybrid quadrupole time-of-flight mass spectrometer, QSTAR® XL Hybrid LC/MS/MS System (Applied Biosystem), in positive ionization, with an ESI of 1800 V, curtain gas 15 unit, CID gas 3 unit. Protein identification was carried out using Mascot search program. Search parameters were as follows: Peptide Mass Tolerance: ± 30 ppm; Fragment Mass Tolerance: ± 0.8 Da; Variable modifications: Oxidation (M); Enzyme: Trypsin; Max Missed Cleavages: 2; Taxonomy: Homo sapiens.

3.7. Western blotting analysis

Validation of mass spectrometry findings was performed by Western blotting analysis. Fifty µg of plasma proteins from pools 1 and 2 were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Immobilon^{TM} P, Millipore). After addition of the blocking mixture, the membranes were incubated with the specific antibodies (all from Santa Cruz Biotechnology, Inc.): anti-apolipoprotein E (mouse, monoclonal), anti-prothrombin; (mouse, monoclonal), anti-voltage-dependent anion-selective channel protein 3 (rabbit, polyclonal); anti-angiotensinogen (rabbit, polyclonal). The signal was detected using anti-mouse horseradish peroxidase-conjugate secondary antibodies and ECL® (Santa Cruz Biotechnology).

4. RESULTS

4.1. Low-abundance proteins enrichment by ProteoMiner™ technology

The combinatorial hexapeptide ligand library chromatography technology was applied to reduce the background noise of plasma samples due to the presence of high-abundance proteins, such as albumin, immunoglobulins, transferrin, etc. Figure 2 shows a representative coomassie-blue stained mono-dimensional gel (2A) and two bi-dimensional silver stained gels of plasma control samples before (2B) and after (2C) treatment with ProteoMiner™ enrichment kit, respectively. As expected, when an equal amount of protein is pre-treated with ProteoMiner, both 1D and 2D PAGE gels show a significantly improved resolution and a greater

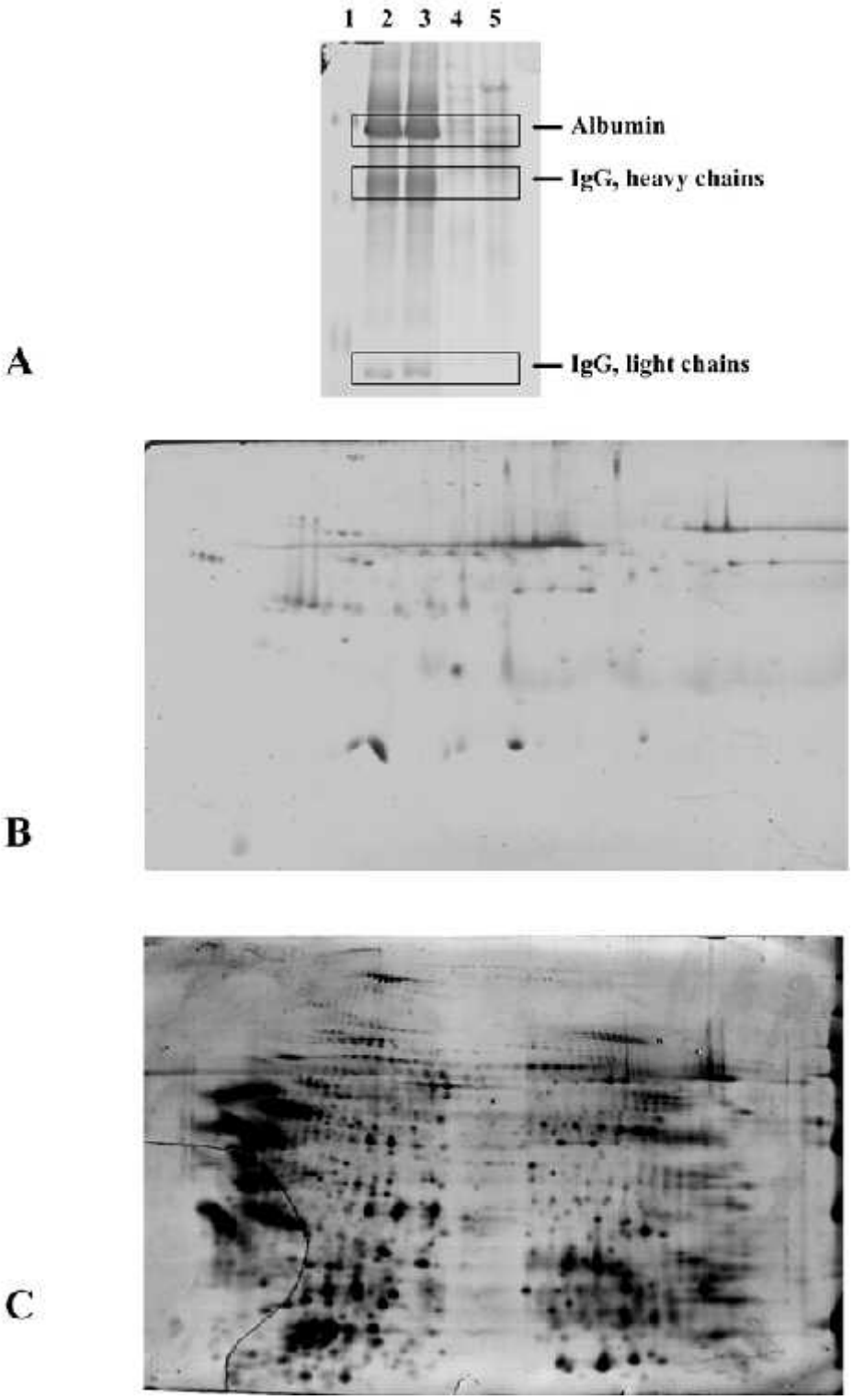


Figure 2. Mono-dimensional (Figure 2A) and two-dimensional-polyacrylamide gel electrophoresis (PAGE) (Figure 2B, and 2C) of plasma samples before and after low-abundance proteins (LAP) enrichment by ProteoMiner^{TM} kit. 2A: Lane 1: MW standard; Lanes 2-3: pool 1 and 2 before (LAP) enrichment; Lanes 4-5: pool 1 and 2 after (LAP) enrichment. 2B: 2D-PAGE pre-enrichment; 2C: 2D-PAGE post enrichment. The figure 2C clearly demonstrates the significant improvement in amount and number of lower represented serum proteins following the removal of the highly abundant ones.

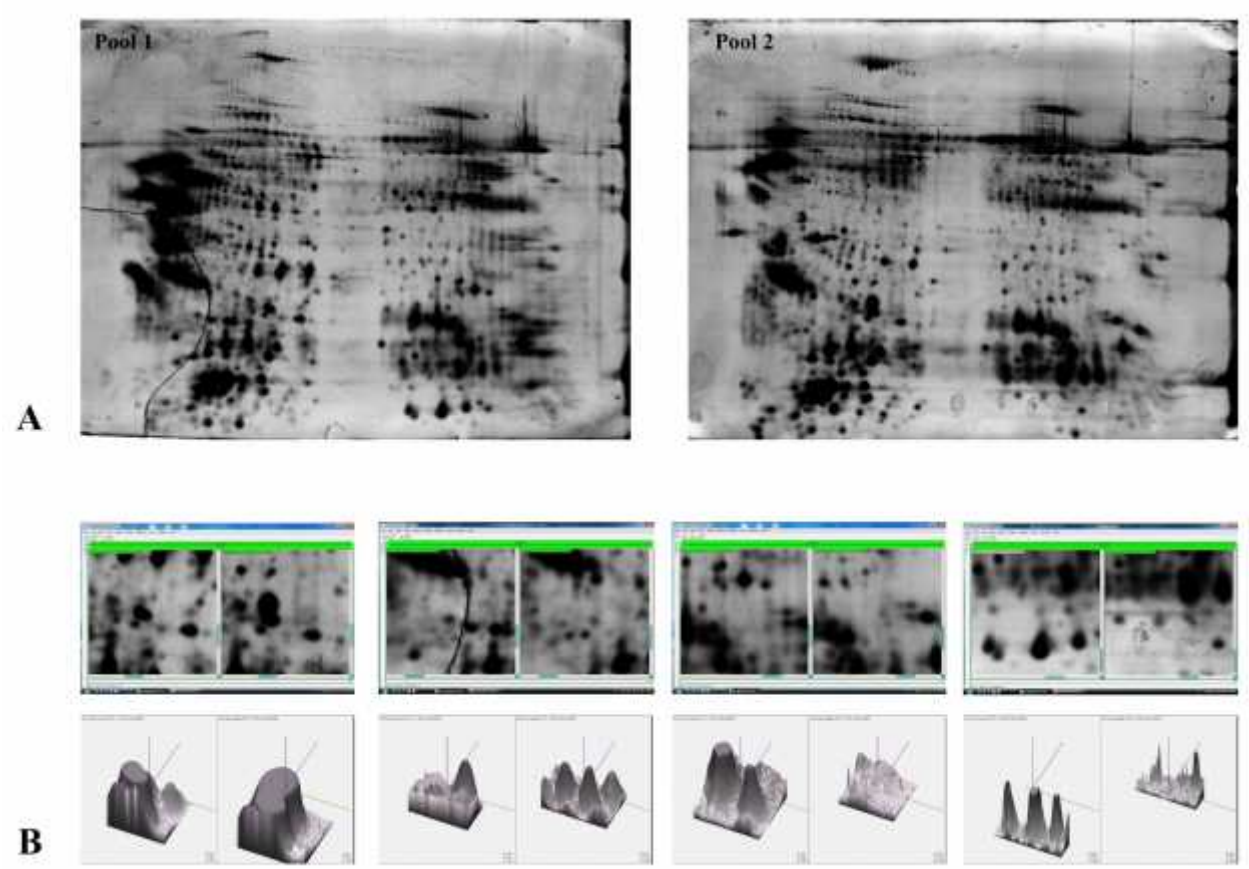


Figure 3. Two-dimensional PAGE analysis of plasma proteins from BS subjects vs. healthy relatives. 3A: Representative 2D-gels in which low-abundance protein enriched plasma from BS individuals (pool 1) and healthy family members (pool 2) were loaded. A significant difference in the number and the intensity of protein spots can be detected by comparing the two gels. 3B: Densitometric analysis analysis of apolipoprotein E, antithrombin-III, prothrombin and alpha-1-antitrypsin performed by the Image Master 2D-Platinum software: the 3D-computational reconstruction of gel spots (lower panels) highlights the differences in intensity between BS-affected and unaffected individuals.

number of spots in samples subjected to combinatorial hexapeptide ligand library enrichment.

4.2. Two-dimensional electrophoresis and protein identification by LC-MS/MS

Each plasma pool was subjected to three distinct runs of 2D-PAGE using 24 cm strips focused in the 4-7 pH range and subsequently separated on a 10% PAGE. Representative gels are shown in figure 3A. Image analysis of the gels, carried out using the Image Master 2D-Platinum software, highlighted the presence of several spots differentially expressed in the two subgroups (pool 1 vs. pool 2). The most representative spots were excised, in-gel digested by trypsin and subsequently subjected to LC-MS/MS. This analysis revealed, in pool 1, an increased expression of the following proteins: apolipoprotein E, prothrombin, vitronectin, complement-factor H, vitamin-D-binding protein, voltage-dependent anion-selective channel protein 3 and clusterin. Conversely, low-abundance proteins enriched plasma proteome of BS individuals showed decreased levels of: Alpha-1-antitrypsin, Fibrinogen and Angiotensinogen were considerably decreased. Interestingly, post-translational modification of antithrombin-III was detected in pool 1. As an example, densitometric analysis of apolipoprotein E, antithrombin-III, prothrombin and alpha-1-antitrypsin is shown in Figure 3B. LC-MS/MS findings on pooled samples were confirmed by performing the analysis on a single patient basis (data not shown).

4.3. Western blotting validation of differentially expressed proteins

Further confirmation with an independent assay was obtained by challenging plasma from the individuals recruited in the present study with antibodies specifically recognizing some of the proteins differentially expressed in BS individuals by means of Western blotting analysis. A representative result is shown in Figure 4. In addition, we demonstrated elevated levels Il-7 measured by ELISA (data not shown).

5. DISCUSSION

The BS is a cardiac disease electrocardiographically characterized by coved-type ST-segment elevations in the right precordial leads and a high

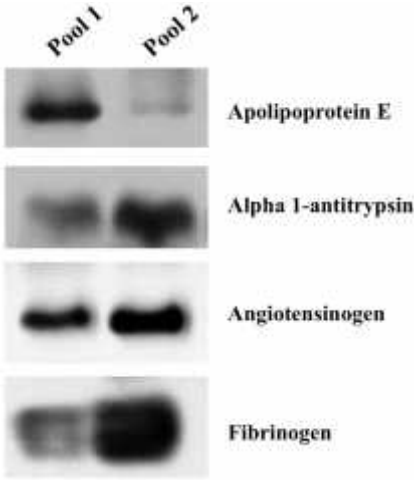


Figure 4. Western blotting analysis of differentially expressed plasma proteins. Validation of 2D-PAGE LC-MS/MS data was performed by challenging plasma from BS individuals and healthy relatives with specific antibodies. A representative analysis, relative to four proteins (apolipoprotein E, alpha-1 antitrypsin, angiotensinogen and fibrinogen) is shown.

risk of sudden cardiac death (SCD) in individuals with a morphologically normal heart (1, 10-12). Diagnosis of BS is not an easy task and is mainly based on the basal ECG, the history of syncope or SCD, and the inducibility of life-threatening tachyarrhythmias during programmed ventricular stimulation. Even though ECG abnormalities have been described as a strong predictor of ventricular arrhythmias, while a basal non-diagnostic ECG is associated to a lower risk of rhythm disturbances, several reports indicate that fluctuations between diagnostic and non-diagnostic ECG are a common feature in BS individuals (9, 13, 14). On the other hand, the detection of gene mutations does not improve significantly risk stratifications of patients diagnosed with a BS because of the incomplete penetrance of the diseased gene (15). Therefore, new, reliable and sensitive diagnostic tools are urgently needed for management optimization of these critical patients.

In this study, we applied a novel, proteomics-driven approach to analyze the plasma proteome of a family in which several members are affected by BS, looking for a "protein signature" that specifically identifies individuals carrying a nonsense mutation in the SCNA gene ($S5CNA^{Q1118X}$) with respect to unaffected, mutation-free, relatives. To this end, a recently developed technology, named ProteoMiner^{TM}, was used to enrich the low-abundance proteome getting rid of the noise-generating highly represented proteins (i.e. albumin, immunoglobulin, transferrin, etc). Plasma samples from $SCNA5^{Q1118X}$ mutation carriers and healthy relatives were divided in two distinct pools (pool 1: BS; pool 2: no-BS) and subjected to two-dimensional gel electrophoresis. Spots differentially expressed between the two groups were subsequently excised, in-gel digested by trypsin and, after elution from the gel, analyzed for protein identification by LC-MS/MS. This approach allowed us to identify several proteins whose levels change significantly depending in circulating plasma upon the respective SCN5A gene status. More specifically, family members carrying the $SCNA5^{Q1118X}$ mutation (regardless of the presence of ECG abnormalities) showed an increased expression of the following proteins: apolipoprotein E, prothrombin, vitronectin, complement-factor H, vitamin-D-binding protein, voltage-dependent anion-selective channel protein 3 and clusterin. On the other hand, decreased levels of alpha-1-antitrypsin, fibrinogen and angiotensinogen were detected in the same pool. Finally, an interesting post-translational modification of antithrombin-III was found in this pool.

Among these proteins, our attention was drawn in particular towards the following: i) apolipoprotein E, a major component of specific lipoproteins called very low-density lipoproteins (VLDL), whose main function is to remove excess cholesterol from the blood and carry it to the liver for processing; a specific version of apolipoprotein E, the e4 allele, is a risk factor for coronary artery disease (16); ii) prothrombin, a blood plasma glycoprotein, essential component of the blood-clotting mechanism. Prothrombin is transformed into thrombin by a clotting factor known as factor X. Mutations in this gene have been linked to venous thromboembolism and susceptibility to ischemic stroke (17, 18); iii) voltage-dependent anion-selective channel protein 3, a major component of the outer mitochondrial membrane which allows diffusion of small hydrophilic molecules and is involved in cell volume regulation and apoptosis (19); iv) alpha-1-antitrypsin, a protease inhibitor belonging to the serpin superfamily. Interestingly, significant elevations of serum alpha-1-antitrypsin have been demonstrated in coronary artery disease and angina cases; v) fibrinogen, a blood-clotting factor known as an important risk factor for coronary heart disease in men and women, with and without pre-existing coronary heart disease (20); vi) antithrombin-III, the primary inhibitor of serine protease coagulation proteins in blood, whose plasma levels (either low or high) are associated with the risk of arterial disease, mainly ischemic heart disease (21); Il-7 elevated was found in serum, which might be integrated in the molecular network of the development of vascular disease.

On the light of the results obtained in the present work, we hypothesize that these proteins might be considered as potential markers for the identification of disease status in BS. Moreover, we postulate that the protein profile obtained from plasma analysis might help, in the near future, the development of innovative, patient-tailored therapeutic strategies for BS in a targeted, highly specific way. Further analysis is undergoing in our laboratory in order to validate these findings in a larger number of cases and to elucidate the pathogenetic role of these proteins in this life-threatening cardiac disease.

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Abbreviations: BS: Brugada Syndrome, ECG: electrocardiogram, 2D-PAGE: two-dimensional

polyacrylamide gel electrophoresis, LC-MS/MS: liquid chromatography-tandem mass spectrometry

Key Words: Brugada syndrome, Serum proteomics, Early diagnosis, Biomarkers, Mass spectrometry

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