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A Proteomic Approach to Investigate Myocarditis

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1. Introduction

Myocarditis is an inflammatory disease of the cardiac muscle which might be related to viral (mainly parvovirus B19 and many others), protozoan (*Borrellia burgdorferi*, *Trypanosoma cruzi*, *Toxoplasma gondi*), bacterial (*Brucella*, *Corynebacterium diphtheriae*, *Gonococcus*, *Haemophilus influenzae*, *Actinomyces*, *Tropheryma whipplei*, *Vibrio cholerae*, *Borrelia burgdorferi*, *Leptospira*, *Rickettsia*), fungal (*Aspergillus*) and other non viral pathogens (Rezkalla SH et al., 2010, Blauwet LA et al., 2010, Cihakova D et al., 2010) infections; It has been reported, however, that this kind of inflammation might be caused by an hypersensitivity response to drugs (Kühl U et al., 2009). The final effect in each case is represented by myocardial infiltration of immunocompetent cells following any kind of cardiac injury.

Myocarditis presents with many symptoms, from chest pain that spontaneously resolves without treatment to cardiogenic shock and sudden death (Kühl U et al., 2010, Taylor CL et al 2010). The major long-term consequence is dilated cardiomyopathy with chronic heart failure (Lv H et al. 2011, Stensaeth KH et al 2011).

Nowadays, diagnostic tools available for this disease are mainly related to general investigations (such as electrocardiography) and analysis of the most abundant serum proteins, whose alteration is related to cardiac pathology, even if it's not specifically connected to myocarditis itself. Here we propose preliminary speculations on the serum proteins profiling (both in the expression level and in the characterization of post translational modifications) and on free peptides identification in myocarditis affected patients (compared to healthy individual), that could be helpful in finding specific markers for this pathology.

As many other inflammatory events this disease involves in fact different kinds of biological macromolecules, here we focus in particular on proteins. More than 50% of total protein content in a cell is post translationally modified. The pattern of Post Translational Modifications (PTMs) on proteins constitute a molecular code that dictates protein conformation, cellular location, macromolecular interactions and activities, depending on cell type, tissue and environmental conditions (Diernfellner AC et al., 2011, Savidge TC,

* Equally contributed to the work.

2011, Hao P et al.,2011). It is very well known that biological function of many proteins is strictly related to the presence of the appropriate set of PTMs. Most importantly, PTMs deregulation might be involved in the development of diseases, in fact, as a consequence of many pathologies, PTMs set might be altered (Dell A et al., 2001, Kim YJ. Et al 1997, Dube DH. Et al., 2005, Granovsky M. Et al 2000).

Proteomics investigations offer all useful tools to deeply investigate this kind of alterations. Two dimensional electrophoresis coupled with software mediated image analysis, affinity chromatographies and especially Mass Spectrometry (MS) present high levels of sensitivity, accuracy and reproducibility and these are all fundamental requirements that this kind of study needs.

As previously described by our group (Carpentieri A, Giangrande C, et al., 2010), serum glycoproteome characterization might be crucial in clinical investigation. In fact we showed that the *N*-glycan profiling of serum glycoproteins extracted from myocarditis affected donors compared to the ones of healthy people shows many peculiarities. We demonstrated that many of the extracted oligosaccharides are in fact incomplete or truncated structures whilst others show a high level of fucosylation, all these results fully matched previously published data about the glycosylation in proteins during chronic inflammation events (Dell A et al., 2001, Kim YJ. Et al 1997, Dube DH. Et al., 2005, Granovsky M. Et al 2000, Carpentieri A, Giangrande C, et al., 2010).

Thanks to the high sensitivity of the most modern analytical techniques, several research groups focus the research at level of peptides rather than at protein level (Taylor-Papadimitriou J. et al., 1994 Amado F et al., 2010, Menschaert G et al. 2010). In fact, these endogenous peptides are referred to as the peptidome. Initially, peptidomic analyses were conducted as a method to study neuropeptides and peptide hormones; these are signaling molecules that function in a variety of physiological processes (Ludwig M. 2011, Colgrave ML et al., 2011). Recent studies have found large numbers of cellular peptides with half-lives of several seconds, raising the possibility that they may be involved in biological functions (Gorman PM et al 2003).

Here we propose preliminary data to show molecular basis of myocarditis using a proteomic approach. The analyses were focused on the serum proteins profiling, namely the study of the expression level and the characterization of glycoproteins involved in the pathology. A classical two-dimensional gel electrophoresis procedure to obtain protein maps and a “gel-free” comparison of the glycoproteomes in healthy and myocarditis human sera by using advanced mass spectrometry were reported. Free peptides identification in myocarditis affected patients (compared to healthy individual), was achieved in order to provide possible specific markers for this pathology.

2. Materials and methods

2.1 Materials

Human serum samples from 8 healthy donors (all Caucasian 4 males and 4 females aged between 60 and 80 years, all other clinical informations were covered by laws on privacy) and from 5 myocarditis affected patients (all Caucasian 3 males and 2 females aged between 60 and 85 years, all other clinical informations were covered by laws on privacy), respectively, have been obtained from the “Servizio Analisi” Policlinico, Napoli. Aliquots of serum samples from different donors were pooled in order to obtain an average overview of glycoforms distribution.

Guanidine, dithiothreitol (DTT), trypsin, α -cyano-4-hydroxycinnamic acid were purchased from Sigma. Iodoacetamide (IAM), tris(hydroxymethyl)aminomethane, calcium chloride and ammonium bicarbonate (AMBIC) were purchased from Fluka as well as the MALDI matrix 2,5-, α -cyano-4-hydroxycinnamic. Methanol, trifluoroacetic acid (TFA) and acetonitrile (ACN) are HPLC grade type from Carlo Erba, whereas the other solvents are from Baker. Gel filtration columns PD-10 are from Pharmacia, the HPLC ones from Phenomenex, whereas the pre-packed columns Sep-pak C-18 are from Waters.

Comassie Brilliant Blue was from Bio-Rad. PNGase F were purchased from Boehringer. Ion exchange resins Dowex H⁺ (50W-X8 50-100 mesh) was provided by BDH. Concanavalin A sepharose resin was purchased from Amersham Biosciences.

2.2 Protein concentration determination

Sera protein concentration was determined by Bradford assay method, using bovine serum albumin (BSA) as standard. Known amounts of BSA were diluted in 800 μ L of H₂O and then mixed to 200 μ L of Comassie Brilliant Blue. 5 different BSA concentrations were determined by measuring absorbance at 595 nm and used to obtain a linear calibration curve. Three different sera dilutions were measured at 595 nm. Absorbance data were interpolated on the calibration curve, allowing the determination of protein concentration in the different samples.

2.3 Serum depletion

The depletion of the most abundant proteins from each serum sample was performed using Multiple Affinity Removal Spin Cartridges from Agilent. The procedure was performed at room temperature according to manufacturer's instructions and then immediately frozen to -20°C.

2.4 Free peptides analysis

300 μ L acetonitrile was added to 100 μ L of serum, incubated at room temperature for 30 minutes and then centrifuged at 13000 rpm. Supernatants were collected and concentrated by vacuum centrifugation (SAVANT) and resuspended in 20 μ L formic acid 0.1%. Samples were desalted by C18 Zip Tip (Millipore) and diluted 100 times prior mass spectrometry analyses.

LC/MS-MS HPLC-Chip/Q-TOF 6520

Peptides were analyzed by a HPLC-Chip/Q-TOF 6520 (Agilent Technologies). The capillary column works at a flow of 4 μ L/min, concentrating and washing the sample in a 40 nL enrichment column. The sample was then fractionated on a C18 reverse-phase capillary column (75 μ m~43 mm in the Agilent Technologies chip) at flow rate of 400 nL/min, with a linear gradient of eluent B (0.2% formic acid in 95% acetonitrile) in A (0.2% formic acid in 2% acetonitrile) from 7% to 60% in 50 min.

Data were acquired through MassHunter software (Agilent Technologies). Proteins identification was achieved by using Mascot software (Matrix science), with a tolerance of 10 ppm on peptide mass, 0.6 Da on MS/MS, and choosing methionine oxidation and glutamine conversion in pyro-glutamic acid as variable modifications.

MALDI-TOF/TOF

Peptides were also analyzed by MALDI-TOF/TOF using a 4800 Plus MALDI-TOF/TOF (Applied Biosystems). Samples were mixed on MALDI plate to the matrix consisting of a

solution of 10 mg/mL α -cyano-4-hydroxycinnamic, whose preparation consisted in the resuspension of α -cyano-4-hydroxycinnamic in water and acetonitrile 10:1 (v/v). The instrument was calibrated using a mixture of standard peptides (Applied Biosystems). Spectra were register even in reflector positive. MS/MS spectra were performed with CID using air as collision gas. Spectra were manually interpreted.

2.5 2D-gel electrophoresis

IEF (first dimension) was carried out on non-linear wide-range immobilized pH gradients (pH 4-7; 7 cm long IPG strips; GE Healthcare, Uppsala, Sweden) and achieved using the EttanTM IPGphorTM system (GE Healthcare, Uppsala, Sweden). Analytical-run IPG-strips were rehydrated with 125 μ g of total proteins in 125 μ l of rehydration buffer (urea 8 M, CHAPS 2%, 0,5% (v/v) IPG Buffer, bromophenol blue 0,002%) for 12 h at 20°C. The strips were then focused according to the following electrical conditions at 20°C: 500 V for 30 min, from 1000 V for 30 min, 5000 V until a total of 15000 Vt was reached. After focusing IPG strips were equilibrated for 15 min in 6 M urea, 30% (vol/vol) glycerol, 2% (wt/vol) SDS, 0.05 M Tris-HCl, pH 6.8, 2% (wt/vol) DTT, and subsequently for 15 min in the same urea/SDS/Tris buffer solution but substituting the 2% (wt/vol) DTT with 2.5% (wt/vol) iodoacetamide. The second dimension was carried out on 12% polyacrylamide gels at 25 mA/gel constant current until the dye front reached the bottom of the gel. MS gel was stained with colloidal comassie.

2.6 Image analysis

Gels images were acquired with an Epson expression 1680 PRO scanner. Computer-aided 2-D image analysis was carried out using the ImageMasterTM 2D Platinum software (GE Healthcare, Uppsala, Sweden). Differentially expressed spots were selected for MS analysis.

In order to find differentially expressed proteins, comassie stained gel image of serum proteins from healthy individual was matched with the one of myocarditis affected patient. The apparent isoelectric points and molecular masses of the proteins were calculated with ImageMaster 2D Platinum 6.0 using identified proteins with known parameters as references.

Relative spot volumes (%V) ($V = \text{integration of OD over the spot area}$; $\%V = V \text{ single spot} / V \text{ total spot}$) were used for quantitative analysis in order to decrease experimental errors. The normalized intensity of spots on three replicate 2-D gels was averaged and standard deviation was calculated for each condition.

2.7 Protein identification by mass spectrometry

In situ digestion

Protein spots were excised from the gel and destained by repetitive washes with 0.1 M NH_4HCO_3 pH 7.5 and acetonitrile. Enzymatic digestion was carried out with trypsin (12.5 ng/ μ l) in 10 mM ammonium bicarbonate buffer pH 7.8. Gel pieces were incubated at 4 °C for 2 h. Trypsin solution was then removed and a new aliquot of the same solution was added; samples were incubated for 18 h at 37 °C. A minimum reaction volume was used as to obtain the complete rehydration of the gel. Peptides were then extracted by washing the

gel particles with 10mM ammonium bicarbonate and 1% formic acid in 50% acetonitrile at room temperature.

Mass spectrometry and protein identification

LC-MS/MS analyses were performed as previously described for free peptides.

Mass spectrometric obtained data were used for protein identification using the software MASCOT that compare peptide masses obtained by MS and MS/MS data of each tryptic digestion with the theoretical peptide masses from all the proteins accessible in the databases (Peptide Mass Fingerprinting, PMF). Database searches were performed in NCB I databank (National Center for Biotechnology Information), restricting the analysis to the pertinent taxonomies. The parameters used for the identification were: tolerance of 10 ppm on peptide mass, 0.6 Da on MS/MS, and cysteine carbamidomethylation as fixed modification. Variable modifications were methionine oxidation, glutamine conversion in pyro-glutamic acid, and asparagine deamidation.

2.8 Boronate affinity chromatography

Glycoproteins were purified using PBA-bound agarose (Sigma-Aldrich, Munich, Germany). 500 µl of sample previously diluted (1 : 1) with equilibration buffer (50 mM taurine/NaOH, pH 8.7, containing 3–10 mM MgCl₂) was incubated with 200 µl of pre-washed immobilized ligand resin for 1 h on ice and with gentle shaking. After transfer of the resin into 1,5 ml eppendorff tubes, the non-binding fraction was collected by low speed centrifugation (10 s, 500 × g). The resin was then thoroughly rinsed with equilibration buffer (six washes of 150 µl each) and 1 N NaCl (three washes of 150 µl each). For final elution of the bound fraction, a total of six washes (150 µl each) with taurine buffer containing 50 mM sorbitol were used. Three successive fractions (150 µl each) were pooled before further analysis.

2.9 Deglycosylation

Glycopeptides were lyophilized, resuspended in 10 mM AMBIC and incubated with PNGase F (5 U), for 12–16 h at 37°C. Deglycosylation was carried out also on unbound peptides, in order to release the glycans not recognized by Boronate affinity chromatography.

3. Results and discussion

3.1 Free peptides analysis

The analyses to investigate the “peptidomic” both in healthy and in pathological samples were accomplished by using a gel-free approach. The peptide component of the eluted fraction was analyzed by tandem mass spectrometry. The stringency of scoring parameters of the MASCOT algorithm minimized the number of false positive identifications. Most MS/MS spectra giving positive hits were derived from doubly and triply charged precursor ions that resulted predominantly in y-ion series.

Triplicate LC-MS/MS analysis of supernatants after ACN precipitation of serum proteins, showed the occurrence of many free peptides in both analyzed sera. As reported in Table 1, the total number of detected and identified peptides was 41. Among these, 9 peptides were unique in the pathologic sample. It should be noted that some peptides were identified in both samples.

m/z	RT	Sequence	Peptide	Protein	H/M ratio
567.95	17.09	QAGAAGSRMNF RPGVLS	(650-666)	Q14624 Inter-alpha-trypsin inhibitor heavy chain H4	H: not detected
513.78	17.70	YYLQGAKIPKPEASFSPR	(627-644)	Q14624 Inter-alpha-trypsin inhibitor heavy chain H4	H: not detected
823.17	22.11	MNFRPGVLSSRQLGLPGPPDVPDH AAYHPF	(658-687)	Q14624 Inter-alpha-trypsin inhibitor heavy chain H4	H: not detected
1005.98	22.78	QLGLPGPPDVPDHAAYHPF	(669-687)	Q14624 Inter-alpha-trypsin inhibitor heavy chain H4	0.68±0.18
757.71	19.96	SRQLGLPGPPDVPDHAAYHPF	(667-687)	Q14624 Inter-alpha-trypsin inhibitor heavy chain H4	0.27±0.09
681.85	22.07	PGVLSSRQLGLPGPPDVPDHAAYHP F	(662-687)	Q14624 Inter-alpha-trypsin inhibitor heavy chain H4	0.18±0.03
576.90	20.69	PGVLSSRQLGLPGPPDVPDHAAYHP FR	(662-688)	Q14624 Inter-alpha-trypsin inhibitor heavy chain H4	H: not detected
379.71	0.55	LAEGGGVR	(28-35)	P02671 Fibrinogen alpha chain	25±3.11
453.24	3.65	FLAEGGGVR	(27-35)	P02671 Fibrinogen alpha chain	2.48±0.57
510.76	11.29	DFLAEGGGVR	(26-35)	P02671 Fibrinogen alpha chain	1.33±0.76
539.27	11.70	GDFLAEGGGVR	(25-35)	P02671 Fibrinogen alpha chain	3.15±1.02
597.77	15.59	SGEGDFLAEGGGV	(22-34)	P02671 Fibrinogen alpha chain	0.85±0.23
603.79	12.57	EGDFLAEGGGVR	(24-35)	P02671 Fibrinogen alpha chain	2.42±0.63
632.30	13.50	GEGDFLAEGGGVR	(23-35)	P02671 Fibrinogen alpha chain	2.72±0.71
655.28	16.03	DSGEGDFLAEGGGV	(21-34)	P02671 Fibrinogen alpha chain	1.23±0.36
675.81	13.67	SGEGDFLAEGGGVR	(22-35)	P02671 Fibrinogen alpha chain	2.32±0.58
690.80	16.13	ADSGEGDFLAEGGGV	(20-34)	P02671 Fibrinogen alpha chain	3.25±0.82
733.33	14.04	DSGEGDFLAEGGGVR	(21-35)	P02671 Fibrinogen alpha chain	1.59±0.12
768.85	14.08	ADSGEGDFLAEGGGVR	(20-35)	P02671 Fibrinogen alpha chain	6.16±1.01
851.71	13.58	SSSYSKQFTSSTSYNRGDSTFES	(576-598)	P02671 Fibrinogen alpha chain	0.65±0.17

m/z	RT	Sequence	Peptide	Protein	H/M ratio
693.06	13.06	SSSYSKQFTSSTSYNRGDSTFESKS	(576-600)	P02671 Fibrinogen alpha chain	M: not detected
733.83	14.48	SSSYSKQFTSSTSYNRGDSTFESKSY	(576-601)	P02671 Fibrinogen alpha chain	M: not detected
619.75	17.89	QGVNDNEEGFF	(31-41)	P02675 Fibrinogen beta chain	3.25±0.89
663.26	16.54	QGVNDNEEGFFS	(31-42)	P02675 Fibrinogen beta chain	1.97±0.41
696.28	17.64	QGVNDNEEGFFSA	(31-43)	P02675 Fibrinogen beta chain	2.31±0.53
404.55	18.90	RIHWESASLL	(1310-1319)	P01024 Complement C3	H: not detected
402.22	14.29	THRIHWESASLLR	(1308-1320)	P01024 Complement C3	H: not detected
445.25	16.98	SKITHRIHWESASLL	(1305-1319)	P01024 Complement C3	H: not detected
415.20	7.60	HWESASL	(1312-1318)	P01024 Complement C3	H: not detected
471.74	16.21	HWESASLL	(1312-1319)	P01024 Complement C3	H: not detected
851.07	22.22	TLEIPGNSDPNMIPDGFNSYVR	(957-979)	P0C0L4 Complement C4-A	H: not detected
1054.53	25.86	DDPDAPLQPVTPLQLFEGR	(1429-1447)	P0C0L4 Complement C4-A	H: not detected
489.96	20.26	RHPDYSVLLLR	(169-180)	P02768 Serum Albumin	0.43±0.17
417.91	16.67	KFQNALLVRY	(426-435)	P02768 Serum Albumin	H: not detected
547.31	16.25	KVPQVSTPTLVEVSR	(438-452)	P02768 Serum Albumin	H: not detected
868.11	21.57	AVPPNNSNAAEDDLPTVELQGVVP R	(14-38)	P00488 Coagulation factor XIII A	2.40±0.84
920.14	21.09	RAVPPNNSNAAEDDLPTVELQGVV PR	(13-38)	P00488 Coagulation factor XIII A	23.60±3.61
837.93	21.39	TAFGGRRAVPPNNSNAAEDDLPTV ELQGVVPR	(7-38)	P00488 Coagulation factor XIII A	91.32±7.39
781.37	17.84	TATSEYQTFFNPR	(315-327)	P00734 Prothrombin	H: not detected
868.46	25.47	TGIFTDQVLSVLKGEE	(86-101)	P02655 Apolipoprotein C-II	H: not detected
572.95	12.91	DALSSVQESQVAQQAR	(45-60)	P02656 Apolipoprotein C-III	H: not detected
803.76	21.14	AATVGSLAGQPLQERAQAWGERL	(210-232)	P02649 Apolipoprotein E	0.02

Table 1. List of free peptides identified by LC-MS/MS. Averaged area of chromatographic peaks from healthy(H) and myocarditis (M) ratio, indicates that some of them were differently represented. Peptide sequences were validated by MALDI-TOF/TOF analyses too.

As a whole, the LC-MS/MS proved to be a very sensitive and reproducible analysis which led to the identification of very weakly present peptides, these data were confirmed by another fragmentation technique, MALDI-TOF/TOF. As Fig. 1 shows, the fragmentation pattern of the peptide -ADSGEGDFLAEGGGVR- (from alpha fibrinogen protein) confirm what we found using LC-MS/MS analysis. Some of these peptides are related to different proteolytic activities on proteins involved in acute phase or inflammatory events, such as myocarditis itself. Among identified peptides, the molecular species , namely, peptide 662-668 from inter-alpha-trypsin inhibitor heavy chain H4 and peptide 1305-1319 from complement C3 correspond to free bioactive peptides, whose activity might be related again to inflammatory events (van den Broek I et al, 2010, ter Weeme M, et al, 2009). Preliminary qualitative and quantitative differences were detected in the analysis of the peptidomas from healthy and pathological samples. In fact, as reported in the Table 1 , averaged area of each peak of all detected peptides resulted to be different, showing that some of them were differently represented in each sample. The above mentioned peptides from complement C3 and inter-alpha trypsin inhibitor were poorly represented in the healthy serum samples, whereas fibrinogen alpha chain peptides were strongly represented in these sera, as well as coagulation factor XIII A peptides.

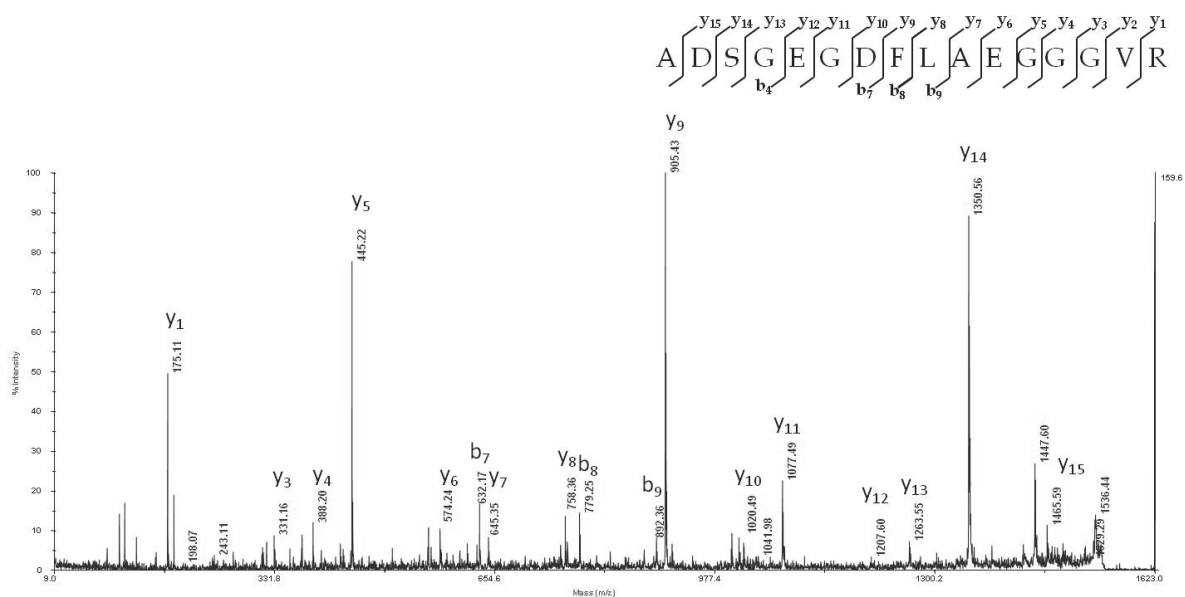


Fig. 1. Positive ion mode MALDI-TOF/TOF fragmentation spectrum of m/z 1536.44 corresponding to peptide 20-35 from fibrinogen alpha chain protein from healthy serum.

3.2 The proteins profile from 2D-electrophoresis is different

After depletion of the most abundant proteins, a two dimensional electrophoretic separation of the two sera samples was performed, thus leading to the construction of 2D protein maps of healthy and myocarditis samples. Each gel was blue comassie stained. Image analysis was performed on the two sets of 2D maps (from healthy control and pathologic sera) clearly showing that the protein profile is quite different, as reported in figure 2. The protein spots which resulted to be differentially expressed in the two samples were then submitted to mass spectral identification. Fig 2 shows all the spots we chose.

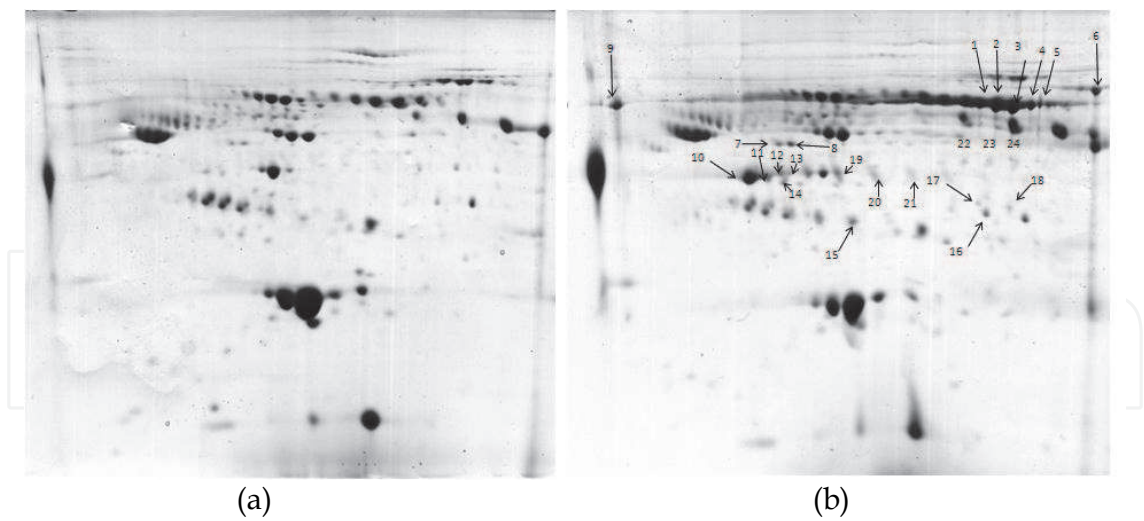


Fig. 2. Two-dimensional electrophoresis gels of healthy (A) and myocarditis affected (B) sera proteins. Arrows and numbers in (B) correspond to numbers of identified spots in Table 2.

It should be underlined that all the spots we further analysed were taken from the pathologic serum and results are summarized in Table 2.

Spot	Accession number	Protein
1	P02790	Hemopexin
2	P02790	Hemopexin
3	P02790	Hemopexin
4	P02790	Hemopexin
5	P02790	Hemopexin
6	P02787	Serotransferrin
7	P01008	Antithrombin III
8	P01008	Antithrombin III
9	P01011	Alpha-1 antichymotrypsin
10	P01024	Complement C3
11	P04196	Histidine rich glycoprotein
12	P01024	Complement C3
13	P00738	Haptoglobin
14	P04196	Histidine rich glycoprotein
15	Q14624	Inter-alpha-trypsin inhibitor heavy chain H4
16	P08603	Complement factor H
17	P08603	Complement factor H
18	P03952	Plasma kallikrein
19	P06727	Apolipoprotein A-IV
20	P00738	Haptoglobin
21	P00738	Haptoglobin
22	P36955	Pigment epithelium-derived factor
23	P36955	Pigment epithelium-derived factor
24	P36955	Pigment epithelium-derived factor

Table 2. List of proteins identified by LC-MS/MS of spots excised from two-dimensional gels of sera taken from myocarditis affected patients.

Proteins excised from the gel were reduced alkylated and, in situ, digested with trypsin. The resulting peptide mixtures were directly analysed by LC-MS/MS according to the peptide mass fingerprinting procedure. MS and MS-MS obtained data were used to search for a non-redundant sequence using the in-house MASCOT software, taking advantage of the specificity of trypsin and of the taxonomic category of the samples. The number of measured masses that matched within the given mass accuracy of 20 ppm was recorded and the proteins that had the highest number of peptide matches were examined.

Thanks to this approach, we could identify many proteins differently expressed in the two sera. Among identified proteins, hemopexin (Dooley H et al., 2010), complement C3 (Adamsson Eryd S et al., 2011, Onat A et al., 2011), plasma kallikrein (Kolte D et al., 2011) are undoubtedly related to inflammatory events and most interestingly they resulted clearly over expressed in the pathologic sample.

A preliminary speculation on identified proteins might involve the function of hemopexin. As shown by literature data (Dooley H et al., 2010, : Mauk MR et al., 2011, Larsen R et al., 2010), hemopexin is a serum protein with the very well known function of scavenging the heme released or lost by the turnover of heme proteins such as haemoglobin or by haemolysis caused by parasitic infection, and thus protects the body from the oxidative damage that free heme can cause (Larsen R et al., 2010). Myocarditis itself it's not related to haemolysis phenomena, but some viral infections may cause it, therefore, finding a very high level of hemopexin in a myocarditis affected patient might be a putative marker of the inflammation itself (quite common are in fact viral myocarditis).

Moreover we found some connections between differently expressed proteins in pathologic serum and identified free peptides; in particular we could detect some free peptides some peptides from proteins Complement C3, Inter-alpha-trypsin inhibitor heavy chain H4, Antithrombin III which results over expressed in pathologic serum, (see Tab 1).

3.3 Boronate affinity chromatography

The third part of this work focus on the investigation of one of the most important post-translational modification, the glycosylation. The importance of investigation of post-translational modifications (PTM) is notably increased in the proteomic era, as they play a critical role in cellular functioning and they vary in response to environmental stimuli, signalling modulators or development of diseases (Laurell E et al., 2011). PTMs can affect biological functions thus playing a critical role in cellular functioning. Moreover, they can vary in response to environmental stimuli, thus finely tuning cellular mechanisms and their deregulation might be involved in the development of diseases. A huge number of different types of PTMs have been identified but only a few are reversible and important for regulation of biological processes (Wu C et al. 2011). The pattern of PTMs on proteins constitute a molecular code that dictates protein conformation, cellular location, macromolecular interactions and activities, depending on cell type, tissue and environmental conditions. Understanding this code is the major challenge of proteomics in post-genomic era. Existing methodologies for PTMs identification essentially rely on specific enrichment procedures able to selectively increase the amount of modified peptides. These procedures have to be integrated with sophisticated mass spectrometric experiments to address the identifications of PTMs. The development of a variety of new technologies for exploring the structures of the sugar chains has opened up a new frontier in the glycomics field. Moreover recent progress in mass spectrometry led to new challenges in glycomics, including the development of rapid glycan enrichment. Recently our group introduced an

easy to handle strategy to give preliminary insights for the comparison of glycoproteomes in healthy and pathological human sera, by using a single Con A affinity chromatography step coupled with mass spectrometry techniques. The strategy led both to the identification of 69 different glycosylation sites within 49 different proteins and to the definition of the glycosylation patterns. Moreover, glycoform distribution in myocarditis and hepatic carcinoma has been reported. The analysis of glycan profiling, once extracted from serum glycopeptides, is essential for comparative studies on different sera samples thus providing a useful tool for the development of screening procedures (Carpentieri A, Giangrande C, et al., 2010).

In this paper, a different simple and rapid procedure to obtain an overview of the glycosylation sites profiling in the two samples was accomplished. This was achieved by enriching for the N-linked glycopeptides resulting from trypsin digestion of sera samples in order to enhance the identification of N-glycosylation sites using LC-MS/MS. The analyses have been carried out by using healthy sera as control.

To reduce the complexity of the whole sample, Boronate affinity purification was rapidly performed in batch after tryptic digestion. Thanks to the vicinal diols binding capacity no discrimination on the basis of the glycan type was performed thus, in a proof of principle, all glycopeptides could be selected. The recovered glycopeptides were then deglycosylated by PNGase F treatment and the peptide mixtures directly analysed by LC-MS/MS.

The analyses were performed on intact serum samples without any pre-purification step or removal of most abundant proteins. The peptide component of the eluted fraction was analysed by tandem mass spectrometry. Similar analyses were carried out on the unbound Boronate fractions, mainly containing non-glycosylated peptides.

The data were then pooled and summarised in Table 3. The results presented here demonstrated that Boronate affinity chromatography on serum tryptic digests is a useful tool to enhance the detection by LC-MS/MS of glycopeptide. Another advantage of the strategy relies on the fact that it was performed on glycopeptides instead of glycoproteins, therefore there were no SDS-PAGE step, no isolation of the individual glycoproteins and no *in situ* digestion. This allowed the detection of less abundant glycopeptides together with the most represented ones, such as those deriving from albumins or immunoglobulins.

As shown in Table 3, all the selected peptides still contained the conserved N-glycosylation motif (Asn-X-Ser/Thr), thus indicating that N-glycosylation peptides were isolated with high selectivity. This analysis led both to the localization of the modification sites and identification of glycoproteins.

The presence of a putative N-glycosylation site was confirmed by the fact that peptides mass was increase of 1 Da, due to the conversion of Asn into Asp after PNGase F incubation. However, some non-specific peptides, namely non-glycosylated peptides, were detected in the eluted Boronate fraction, and identified as belonging to most abundant proteins like albumin.

Spontaneous deamidation seems rather unlikely for generating the results presented, although it cannot be excluded completely. Most MS/MS spectra giving positive hits were derived from doubly charged precursor ions that resulted predominantly in y-ion series.

As a whole, using Boronate affinity approach we could confirm the previously identified glycosylation sites based on ConcanavalinA enrichment and 5 more glycosylation sites were identified thus refining previous data (Carpentieri A, Giangrande C, et al., 2010) on myocarditis glycoproteome.

	Protein	Sequence	Peptide
P01011	Alpha-1-antichymotrypsin	YTGN*ASALFILPDQDKH,M	268-283
P02763	Alpha-1-acid glycoprotein	SVQEIQATFFYFTPN*KTEDTIFLR ^{H,M} QDQCIYN*TTYLNVQR ^{H,M}	58-81 87-101
P01009	Alpha-1-antitrypsin	YLGN*ATAIFFLPDEGKH,M QLAHQSN*STNIFFSPVSIATAFAMLSL GTKH ADTHDEILEGLNFN*LTEIPEAQIHEGF QELLRH,M	268-283 64-93 94-125
P01023	Alpha-2-macroglobulin	SLGNVN*FTVSAEALSEQELCGTEVPS VPEHGRKH	864-886
P43652	Afamin	DIENFN*STQKH,M YAEDKFN*ETTEKH	28-37 396-407
P01008	Antithrombin III	LGACN*DTLQQLMFVKH,M SLTFN*ETYQDISELVYGAKH,M	124-139 183-201
P04114	Apolipoprotein B-100	FN*SSYLQGTNQITGRH,M FVEGSHN*STVSLTTKH AEEEMLEN*VSLVCPKM YDFN*SSMLYSTAKM	1522-1536 3405-3419 27-41 3462-3474
P05090	Apolipoprotein D	ADGTVNQIEGEATPVN*LTEPAKM	83-104
P02749	Apolipoprotein H	VYKPSAGN*NSLYRH LGN*WSAMPSCKM	155-167 251-261
O75882	Attractin	IDSTGN*VTNELRH,M GPVKMPSQAPTGNFYQPPLLN*SSMC LEDSRH	411-422 1023-1052
P00450	Ceruloplasmin	EHEGAIYPDN*TTDFQRH,M EN*LTAPGSDSAVFQEQTTRH,M	129-144 396-414
P08603	Complement factor H	ISEEN*ETTCYMGKH MDGASN*VTCINSRH,M IPCSQPPQIEHGTIN*SSRM SPDVIN*GSPISQKH	907-919 1024-1036 868-885 212-224
P10909	Clusterin	LAN*LTQGEDQYYLRH,M	372-385
Q8IWV2	Contactin-4	LN*GTDVDTGMDFRM	64-76
P05156	Complement factor 1	FLNN*GTCTAEGKH,M	100-111
P01024	Complement C3	TVLTPATNHMGN*VTFTIPANRH	74-94
P0C0L4	Complement C4-A	GLN*VTLSSSTGRH,M	1326-1336
P02748	Complement component C9	AVN*ITSENLIDDVVSLIRH,M	413-430
Q14517	Protocadherin-fat 1	QVYN*LTVRAKDKM FSMDYKTGALTVQN*TTQLRSRM	994-1005 1930-1950
P02765	Alpha-2-HS-glycoprotein	VCQDCPLLAPLN*DTRH,M AALAAFNAQNN*GSNFQLEEISRH	145-159 166-187
Q03591	Complement factor H related protein 1	LQNNENN*ISCVERH	120-132

	Protein	Sequence	Peptide
Q13439	Golgin subfamily A member 4	HN*STLKQLMREFNTQLAQKH	1990-2008
P02790	Hemopexin	SWPAVGN*CSSALRH,M ALPQPQN*VTSLLGCTHH,M	181-193 447-462
P00738	Haptoglobin	VVLHPN*YSQVDIGLIKH,M MVSHHN*LTTGATLINEQWLLTTAKH ,M NLFLN*HSEN*ATAKH,M	236-251 179-202 203-215
P04196	Histidine rich glycoprotein	VEN*TTVYYLVLDVQESDCSVLSRH VIDFN*CTTSSVSSALANTKH,M	61-83 121-139
P05155	Plasma protease C1 inhibitor	VGQLQLSHN*LSLVILVPQNLKH,M	344-364
P01857	Ig alpha-1 chain C region	LSLHRPALEDLLLGSEAN*LTCTLTGL RH	127-153
P01859	Ig gamma-2 chain C region	TKPREEQFN*STFRH TPLTAN*ITKH	168-180 200-208
P01860	Ig gamma-1 chain C region	EEQYN*STYRH,M	136-144
P01591	Immunoglobulin J chain	EN*ISDPTSPLRH,M	48-58
P40189	Interleukin-6 receptor beta	QQYFKQN*CSQHESSPDISHFERM	812-833
P56199	Integrin alpha-1	SYFSSLN*LTIRM	1096-1106
P29622	Kallistatin	DFYVDEN*TTVRH	232-242
P01042	Kininogen-1	LNAENN*ATFYFKH,M	389-400
P11279	Lysosome-associated membrane glycoprotein 1	DPAFKAAN*GSLRM	314-325
P01871	Ig mu chain C region	YKN*NSDISSTRH GLTFQQN*ASSMCVPDQDTAIRM	44-54 204-223
Q9HC10	Otoferlin	NEMLEIQVFN*YSKVFSNKH,M	59-76
Q5VU65	Nuclear pore membrane glycoprotein 210-like	EVVVN*ASSRH	1551-1559
Q9Y5E7	Protocadherin beta 2	ETRSEYN*ITITVTDFGTPRM	414-432
P36955	Pigment epithelium derived factor	VTQN*LTLIEESLTSEFIHDIDRH	282-303
P27169	Serum paraoxanase/arylester ase1	HAN*WTLTPLKH	250-259
P49908	Selenoprotein P	EGYSN*ISYIVVNHQGISSRH	79-97
Q9Y275	Tumor necrosis factor ligand superfamily member 13B	CIQNMPETLPN*NSCYSAGIAKM	232-252

	Protein	Sequence	Peptide
P40225	Thrombopoietin	IHELLN*GTRGLFPGPSRRM	250-267
P02787	Serotransferrin	QQQHILFGSN*VTDCSGNFCLFRH,M CGLVPVLAENYN*KSDNCEDTPEAGY FAVAVVKM	622-642 421-452
P07996	Thrombospondin 1	VVN*STTGPGEHLRH,M	1065-1077
P04004	Vitronectin	NN*ATVHEQVGGPSLTSDLQAQSKM	85-107
P25311	Zinc-alpha-2-glycoprotein	DIVEYYN*DSN*GSHVLQGRH,M	100-117
P02766	Transthyretin	ALGISPFHEHAEVVFTAN*DSGPR ^H	101-123

Table 3. LC/MSMS analysis for the identification of glycosylation sites, H indicates peptides deriving from healthy serum and M indicates the ones from myocarditis serum.

4. Conclusions

In biomedical applications, a comparative approach is usually employed to identify proteins that are up and down regulated in a disease specific manner for use as diagnostic markers or therapeutic targets. This report represents an overview of the investigation at molecular level of myocarditis by using a proteomic approach .

Serum proteins (including the N-glycosylation sites profiling) and glycoproteins and free peptides occurring in human sera from healthy donors were compared to the ones from myocarditis patients. This procedure, allowed the identification of several N-glycosylation sites by a single-step proteomic approach, contemporarily probing an entire complex sample by LC-MS/MS. Thanks to the depletion of the serum most abundant proteins, we could detect some of the very weakly represented free peptides, whose presence is connected to the pathology itself. The high resolution, the sensitivity and the reproducibility of the used techniques led to the identification of some up regulated proteins in the serum from a myocarditis affected patient, all these proteins are connected to inflammatory events and one in particular (hemopexin) opens the way to new speculations in serum proteins as a specific marker for pathologic state.

Finally, this proteomic approach represents a new opportunity for therapeutics and early diagnostics, for the screening of proteic biomarkers in pathological status. Finding a biomarker molecule that precisely indicates certain kind of pathology, is something quite difficult to achieve since it requires a huge background in many different fields of clinical investigation. Here we contribute with putative diagnostic species that could really be helpful for an early diagnosis myocarditis event.

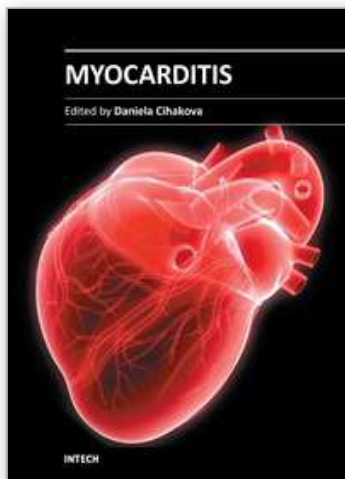
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Myocarditis

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Myocarditis, the inflammation of the heart muscle, could be in some cases serious and potentially fatal disease. This book is a comprehensive compilation of studies from leading international experts on various aspects of myocarditis. The first section of the book provides a clinical perspective on the disease. It contains comprehensive reviews of the causes of myocarditis, its classification, diagnosis, and treatment. It also includes reviews of Perimyocarditis; Chagas's™ chronic myocarditis, and myocarditis in HIV-positive patients. The second section of the book focuses on the pathogenesis of myocarditis, discussing pathways and mechanisms activated during viral infection and host immune response during myocarditis. The third, and final, section discusses new findings in the pathogenesis that may lead to new directions for clinical diagnosis, including use of new biomarkers, and new treatments of myocarditis.

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