Differential expression of proteins with heparin affinity in patients with rheumatoid and psoriatic arthritis: a preliminary study

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Abstract

Objectives

Using proteomic approach in this study, we sought to identify proteins with heparin affinity associated with rheumatoid arthritis (RA), psoriatic arthritis (PsA) and non-inflammatory arthritis (NIA).

Methods

Plasma samples from adult RA, PsA and NIA patients, 20 of each, were collected. After enrichment of proteins with heparin affinity, SDS-PAGE and in-gel digestion with trypsin were performed. Peptides were concentrated, micro-purified, separated and measured by nano-scale HPLC system coupled to a mass spectrometer. Peak lists were generated from raw spectra and searched against human complete proteome set by MaxQuant software. Statistical analysis of protein relative expression levels was done in IPython interactive Python shell using NumPy and Matplotlib libraries. Individual protein impact on the whole dataset correlation was done by excluding one protein at a time and calculating the correlation coefficient of remaining data points.

Results

Three hundred and eighty-four different proteins were identified keeping false discovery rate to 1%, from which 163 were identified in all three conditions. The plasma proteome showed a good correlation between rheumatoid (RA) and psoriatic arthritis (PsA). Out of 10 proteins whose impact on the correlation coefficient fell outside of two standard deviations from the mean, four were up-regulated (complement factor I, complement component C8 beta, glyceraldehyde-3-phosphate dehydrogenase and inter-alpha-trypsin inhibitor heavy chain H1), and two were down-regulated (immunoglobulin heavy chain V-III region BRO, and immunoglobulin J chain), both in PsA and RA by a similar ratio when compared to NIA. The remaining four proteins (Serpin A11, complement factor H-related protein 5, cartilage acidic protein 1 and coagulation factor IX) were down-regulated in PsA and up-regulated in RA when compared to NIA.

Conclusion

We found differently expressed proteins in patients with inflammatory and non-inflammatory rheumatic conditions. Out of 384 proteins with heparin affinity four proteins should be further validated as potential diagnostic biomarkers in patients with RA and PsA.

Key words

liquid chromatography, mass spectrometry, proteomics, psoriatic arthritis, rheumatoid arthritis

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Introduction

Rheumatoid arthritis (RA) and psoriatic arthritis (PsA) are among most prevalent and disabilitating inflammatory rheumatic diseases. RA is characterised by a symmetrical destructive polyarthritis, while psoriatic arthritis is classified in a group of spondyloarthropathies (SpA) with much more variable features including oligoarticular or polyarticular arthritis, enthesitis, dactylitis and/or spondylitis (1, 2). The level of inflammation in an affected joint is closely associated with joint damage (3). During that process, chondrocytes actively synthesise new extracellular matrix and proteolytic enzymes responsible for tissue remodelling and catabolic breakdown of cartilage. Molecules produced and released during inflammatory and degradation process of articular tissue may be detectable in blood and could represent a novel biomarker. The identification of distinctive biomarkers related to arthritis have a great clinical importance for understanding the molecular basis of the disease, diagnosis and treatment.

Several studies explored molecular mechanisms of inflammatory rheumatic diseases (4, 5). Recently, in the serum of patients with established SpA and RA a similar pattern of cytokine concentration has been found, with the exception of higher levels of interleukin-21 in the synovial fluid of RA patients (6).

Also, in RA patients, in addition to rheumatoid factor antibodies, new antibodies targeting citrullinated proteins have been identified as a specific biomarker, which is in contrast with spondyloarthropathies, where these antibodies are found in a small percentage (7, 8). Since PsA is often presented with heterogeneous signs and symptoms, sometimes indistinguishable from other rheumatic diseases, including RA, the aim of our study was to identify new potential biomarkers specific for PsA or RA that might enable the diagnosis prior to specific clinical manifestations, help in understanding pathophysiology of these conditions and eventually pave the way towards new ideas regarding their treatment. We used proteomic analysis of plasma proteins with affinity to heparin, based on our previous work on the evaluation of plasma growth factors in various disease conditions (9, 10).

Materials and methods

Patients

Consecutive patients at the Department of Rheumatology, Physical and Rehabilitation Medicine in the Clinical Hospital Centre "Sisters of Charity", Zagreb, Croatia, were included in the study: RA (age 21–62), PsA (age 25–65) and NIA (non-inflammatory arthritis) (age 24–65), respectively. There were 20 adult patients in each group and the diagnosis was established by two experienced rheumatologists (SG, FG). Overall, the number of patients with RA and PsA that were treated with disease-modifying anti-rheumatic drugs (DMARDs) was 14 and 7, respectively. Systemic glucocorticoids of a stabile dose in the previous 3 months (up to 10 mg of prednisolone per day or equivalent dose) were used in 13 patients with RA and 5 patients with PsA. None of the subjects was on treatment with biological drugs. The study was in compliance with the Helsinki Declaration and was approved by the hospital Ethics Committee (11). All participants signed an informed consent prior to the inclusion into the study.

Plasma collection

Fresh fasting blood samples were drawn into syringes containing 3.8% sodium citrate to form an anticoagulant to blood ratio (v/v) 1:9. Plasma was obtained by centrifugation (15 min at 3000 x g) and aliquots of each adult blood sample were pooled and stored at -80°C until analysis.

Heparin affinity column purification

Pooled plasma of patients with RA, PsA and NIA (60 ml for each, respectively), from which 1 mL was collected for further analysis, was diluted twofold with 10 mM sodium phosphate buffer (pH#7) and applied to a HiTrap Heparin HP column (GE Healthcare). Flow through fraction was also collected for further analysis. Bound proteins were eluted from the column with 10mM sodium phosphate buffer (pH#7) containing 1M sodium chloride. Eluted fractions

along with plasma sample and flow through fraction were precipitated with saturated ammonium sulphate (SAS) in a final concentration of 35% (w/v). Precipitated samples were centrifuged (10 min at 12 000 x g) and supernatant was discarded. Proteins were dissolved in 5% (w/v) SDS and their concentration was measured using BCA method (12). For further SDS-PAGE separation protein sample volume was calculated and normalised to a 75 μg per well.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and in-gel digestion

Prepared protein samples were separated by SDS-PAGE using NuPAGE 10% Bis-Tris gel (Invitrogen), as instructed by the manufacturer. Subsequently, gel was stained with a Coomassie Brilliant Blue for 30 minutes and afterwards the gel was destained leaving only protein bands visible. Each gel lane was cut into 12 slices and placed in a tube containing digestion buffer. The slices were then subjected to in-gel reduction, alkylation and trypsin digestion (13). Briefly, proteins were reduced with 10 mM dithiothreitol (DTT) in 100 mM ammonium bicarbonate (ABC) for 30 minutes at 56°C, then alkylation was done with 55 mM iodoacetamide in 100 mM ABC for 20 minutes at room temperature in dark. Slices were washed with 100 mM ABC, acetonitrile (ACN) mix 1:1 (v/v) for 30 minutes. Gel pieces were further dried in a vacuum centrifuge and then rehydrated with trypsin (Promega) (13 ng/ul in 50 mM ABC) for 30 minutes at 4°C. Gels were then covered with 50 mM ABC and incubated overnight at 37°C. In between every step gel pieces were shrunk with ACN. Tryptic peptides were extracted from gel pieces stepwise with 30% (v/v) ACN, 3% (v/v) triflouroacetic acid; 80% (v/v) ACN, 0.5% (v/v) acetic acid and 100% (v/v) ACN. ACN was evaporated by vacuum centrifugation.

Peptide preparation for mass spectrometry

Peptides were concentrated and micropurified, with the stop-and go extraction tips (StageTips) (14). Briefly, C18 tips were wetted with methanol and then conditioned with 0.5% (v/v) acetic acid (AcOH). Peptides were acidified and loaded to such conditioned tips. Salts were washed with 0.5% (v/v) AcOH and peptides were eluted with 80% (v/v) ACN, 0.5% (v/v) AcOH. ACN was evaporated from eluate by vacuum centrifugation.

Liquid chromatography – mass spectrometry (LC-MS)

Peptides were separated and then measured by nano-scale high performance liquid chromatography (HPLC) system Easy-nLC (Thermo Scientific) coupled to LTQ-Orbitrap Discovery mass spectrometer (Thermo Scientific) through a nano-electrospray LC-MS interface (Thermo Scientific) (15). Microcolumn for peptide separation was made in-house by methanol slurry packing of ReproSil-Pur C18-AQ 3 μm silica particles (Dr Maisch GmbH, kindly given to us by Dr Boris Macek) into 75 μm ID x 15 cm long tapered fused silica capillary (NewObjective) (16). Peptides were loaded onto microcolumn in solvent "A" (0.5% (v/v) AcOH in ultrapure water, Merck) and eluted with a 70-minute linear gradient of 2 – 40% solvent "B" (80% CAN (v/v), 0.5% (v/v) AcOH in ultrapure water) at a flow rate of 250 nL/min.

Mass spectrometer was automatically switching between mass spectrometry (MS) and tandem mass spectrometry (MS/MS) scan type in data dependent mode. Each measurement cycle consisted of a full MS scan and MS/MS scan of up to ten most-intense ions. Full MS scans (m/z from 300 to 2000) were acquired in the Orbitrap analyser at a resolution of 30.000 with accumulation target value of 1×10^6 charges and internal calibration by lock mass. MS/MS scans were acquired in parallel to MS scans by linear ion trap, excluding singly charged ions and dynamically excluding previously selected ions for 90 s at 3×10^5 charge accumulation target value and default CID settings.

MS data processing and database search

Peak lists were generated from raw spectra using the Quant module of MaxQuant software, version 1.1.1.14 using default parameters (17). Peak lists were searched against human complete proteome set (Uniprot, ver. 2011_02) by Andromeda module of MaxQuant package using default search parameters (18). Label-free quantification data were generated from protein raw total intensities produced by MaxQuant. Briefly, protein raw total intensities are sum of extracted ion intensities of all signals corresponding to individual peptides, which belong to the same protein. Individual protein raw intensities are then normalised by the number of observable tryptic peptides to be able to compare quantitative data from proteins with different number of possible tryptic peptides. Normalised protein intensities were normalised once more by the total protein intensity per sample, to compensate for the sample loading variability. Fold change data were calculated as base two logarithms of normalised protein intensity ratios for PA and RA towards NIA (19).

Statistical analysis

Statistical analysis of protein relative expression levels was done in IPython interactive Python shell using NumPy and Matplotlib libraries. Individual protein impact on the whole dataset correlation was done by excluding one protein at a time, and calculating correlation coefficient of the remaining data points. This transformation has enabled us to evaluate individual protein impact on whole dataset correlation.

Results

Protein concentration measured in pooled plasma showed no difference between experimental groups. Heparin affinity purification was used to isolate targeted proteins in plasma sample from RA and PsA patients. To visualise the difference between heparin bound, plasma and flow through fraction protein samples they were separated on a polyacrylamide gel. No visual difference between RA and PsA samples were observed on gels. Whole plasma and flow through fractions showed similar protein bands, while heparin column depleted most of highly abundant proteins, like albumin, and enriched the sample with targeted low abundant pro-

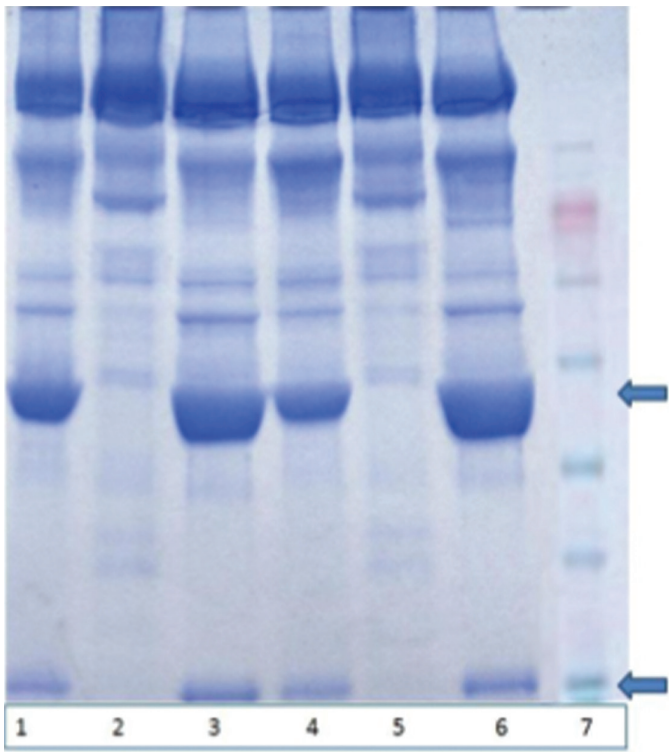

Fig. 1. One dimensional SDS gel protein separation of samples from two patient experimental groups (lane 1–3 RA, lane 4–6 PsA, lane 7 marker). Lanes 1 and 4 represent plasma, while lanes 2 and 5 show proteins bound to heparin column. Flow through fraction is visualised in lanes 3 and 6. Arrows indicate highly abundant proteins which were removed by heparin column purification.

teins (Fig. 1). Column binding capacity was not diminished by a total of 60mL of plasma, as proteins of interest did not appear in the flow through fraction.

Using GeLCMS proteomics approach, we have identified 383 different proteins, keeping false discovery rate (FDR) to 1%, from which 163 of them were found in all three conditions (supplementary Table I).

Taking NIA as a control, we compared PsA and RA protein fold changes (Fig. 2). There was a correlation between PsA and RA protein fold changes, indicating that proteins from PsA and RA were at similar levels as compared to NIA. Proteins at the upper right and lower left corners in Figure 2 were up- or down-regulated in PsA and RA as compared to NIA. Proteins at the lower right corner were lowered in PsA and raised in RA as compared to NIA, and represent those that differentiate PsA from RA.

To quantify the impact of individual proteins on the whole dataset, we have calculated the correlation coefficient between PsA and RA protein fold change towards NIA, excluding one by one protein from the dataset (Fig. 3). The mean correlation coefficient was just below 0.6 and there were 10 proteins whose impact on the correlation coefficient fell outside of two standard deviations from the mean. The correlation coefficient absolute shift from the mean value was used as a marker size in Figure 2.

Six of the top ten proteins have beneficiary effect on the correlation, which means that they were up-regulated or down-regulated in PsA and RA by similar ratio as compared to NIA (Table I). Complement factor I (P05156), complement component C8 beta (P07358), glyceraldehyde-3-phosphate dehydrogenase (P04406), and inter-alpha-trypsin inhibitor heavy chain H1 (P19827) were up-regulated (upper right corner of Fig. 2). Immunoglobulin heavy chain V-III region BRO (P01766) and immunoglobulin J chain (P01591) was down-regulated (lower left corner of Fig. 2).

Four proteins had a negative impact on the correlation coefficient and were down-regulated in PsA and up-regulated in RA: Serpin A11 (Q86U17), complement factor H-related protein 5 (Q9BXR6), cartilage acidic protein 1 (Q9NQ79) and coagulation factor IX (P00749) (lower right corner of Fig. 2).

Table I. Ten most influential proteins identified by this preliminary study. ID: MaxQuant identification number; PEP: posterior error probability.

ID	Uniprot accession key	Protein descriptions	PEP	Mol. weight [kDa]	Unique peptides		
					NIA	PsA	RA
218	P19827	Inter-alpha-trypsin inhibitor heavy chain H1	<2.2E-308	101.39	3	13	20
167	P07358	Complement component C8 beta chain	1.39E-65	67.046	2	17	13
140	P04406	Glyceraldehyde-3-phosphate dehydrogenase	1.67E-177	36.053	1	9	8
69	P01591	Immunoglobulin J chain	7.56E-72	18.098	6	1	5
91	P01766;P01777; P01767;P01763	Ig heavy chain V-III region BRO; Ig heavy chain V-III region TEI; Ig heavy chain V-III region	7.63E-94	13.227	2	1	0
150	P05156	Complement factor I	1.77E-21	65.75	1	7	5
53	P00740	Coagulation factor IX	8.68E-42	51.778	4	2	5
360	Q9BXR6	Complement factor H-related protein 5	1.57E-31	64.419	2	3	5
340	Q86U17	Serpin A11	2.23E-15	46.989	1	1	4
367	Q9NQ79;Q9NQ79-2;Q9NQ79-3	Cartilage acidic protein 1	1.31E-35	71.42	2	3	6

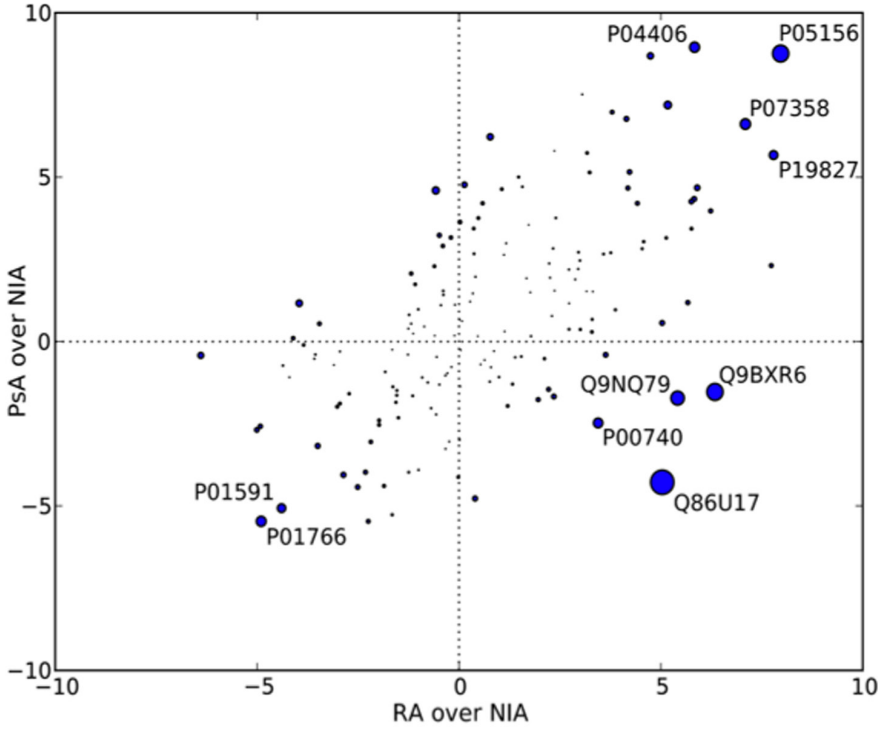

Fig. 2. Protein biomarker candidates discriminating PsA and RA from NIA (upper right and lower left quadrant) and PsA from RA (lower right quadrant). Base two logarithm of PsA over NIA fold change was plotted against base # two logarithm of RA over NIA fold change. Marker size is proportional to impact protein has on cross correlation of dataset. (Fig. 3) Top ten discriminating proteins are annotated with Uniprot accession key.

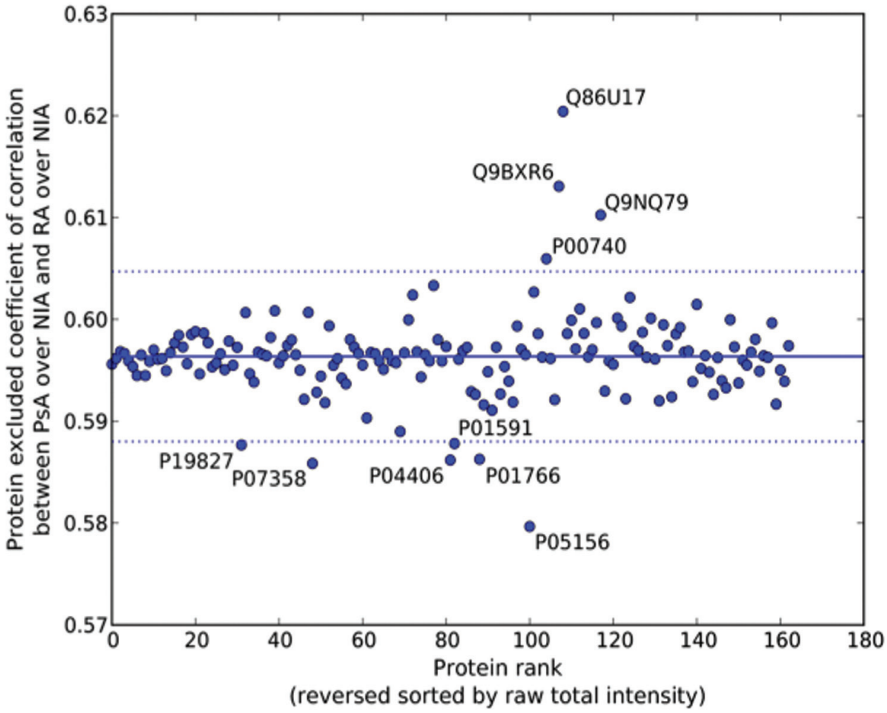

Fig. 3. Influence of individual proteins on the coefficient of correlation between PsA and RA fold change toward NIA. The correlation coefficient was calculated between PsA and RA protein fold changes towards NIA excluding one by one protein from the dataset. Proteins were ranked by raw total intensity in reversed order, and the top 10 proteins with the highest impact on the correlation coefficient were annotated by the Uniprot accession key. Dotted lines denote two standard deviation windows, and solid line denotes mean value.

Discussion

In this study, we used GeLCMS proteomics approach to characterise differences in the heparin bound protein profile in patients with inflammatory rheumatic conditions (RA and PsA) compared to non-inflammatory arthritis (NIA). Among 10 most influential proteins we have identified in this preliminary study, 4 were differently expressed in PsA as compared to RA.

Among them we identified SERPIN A11, a serine proteinase inhibitor. Previously it was confirmed (by RT-PCR) that several proteinases and proteinase inhibitors were expressed at a higher level in cartilage including: SERPIN A1, SERPIN A3, matrix metalloproteinase 3 (MMP3), the proprotein convertase inhibitor PCSK1N and the protease HTRA1 (20, 21). It is tempting to speculate that serpins together with MMPs counter act in the homeostasis of cartilage matrix. Our results indicate for the first time that in patients with RA SERPIN A11 is up-regulated in plasma when compared to patient with PsA, which might be used as a differentiation marker between those two conditions. Beside the main function of serpins (22), SERPIN A11 may indicate a more pronounced cartilage injury in RA, where increased amount of serpin might protect against matrix degradation by inhibition of targeted proteinases. This might include SERPIN A11 as a novel potential biomarker for early diagnosis of RA, which could enable a distinction between RA and PsA.

In favour of greater damage of cartilage in RA is the up-regulation of glycosylated extracellular matrix molecule cartilage acidic protein 1 (CRTAC1), which is secreted by chondrocytes and together with collagen type II is a marker for cultured chondrocytes (23). Opposite of that in patients with PsA serum level of cartilage oligomeric matrix protein (COMP) was significantly elevated in patients with active disease (24). In our previous work, we showed that presence of CRTAC-1 in the plasma of patients with an acute fracture could indicate a normal development of cartilaginous callus formation within the first week after the fracture, but it

could also indicate a concomitant joint cartilage injury (9, 25). Since CRTAC1 is expressed by all chondrocytes in articular cartilage as detected by *in situ* hybridisation, the protein seems to accumulate preferentially close to the tide mark in the deep zone of articular cartilage (26). Future studies should explore the correlation between the plasma CRTAC1 and the damage of cartilage in RA and PsA.

In RA complement-binding, complement-derived inflammatory mediators or immune complexes are present within joint and may contribute to local tissue damage (27). The complement activation profile showed that in RA complement activation is induced mainly by an alternative pathway (28, 29). PsA patients with a moderate and severe disease showed elevated C3 and C4 components of the complement system as an important hallmark of inflammation (30). In RA we detected in human plasma up-regulation of complement factor H-related protein 5 (FHR-5), a member of the human factor H (fH) protein family that are structurally and immunologically related proteins which are important negative regulators of an alternative pathway of complement activation (31). Previous studies have already shown in the synovial tissue an upregulation of the factor H (32), where they indicate an important anti-inflammatory and protective effect. FHR-5 is known to be involved in protein binding and complement-regulatory activity and it is unique among the FHR proteins to bind C3b, heparin and CRP (33). Measuring the level of complement factor H-related protein 5 in plasma may be useful in therapy follow-up for better assessment of disease staging.

As a chronic disease of unknown etiology RA affects multiple systems of the human body. Among them manifestation abnormalities in coagulation parameters have been reported and some authors suggested that RA is associated with increased thrombotic potential, reflected by increased levels of thrombin markers such as thrombin-antithrombin complexes and prothrombin fragments 1+2. Elevated fibrinogen, von Willebrand factor (VIII) and D-dimer were observed in RA patient even during remission (34-37). Several studies showed that RA with no concomitant cardiovascular disease alters the kinetics of the thrombin generation and the changes are largely dependent on plasma coagulation factors, namely 100% higher FVIII was counteracted to some extent by the increased free TFPI (38). Blood viscosity ascending and enhancement of the aggregation activity of thrombocytes was revealed in patients with RA accompanied by systemic manifestations and changes in laboratory parameters, particularly the immune shift (39). Platelet hyperreactivity without affection of coagulation parameters was also noted in patients with PsA in active phase of the disease (40). In this study, we also showed changes in the composition of plasma coagulation factor IX which was up-regulated in patients with RA and down-regulated in patients with PsA. Coagulation factor IX plays an important role in the blood coagulation cascade. After being activated, factor IX forms a complex with calcium ions, membrane phospholipids and coagulation factor VIII to activate coagulation factor X most important for thrombin activation. This result can be useful in the mechanism of thromboembolic cardiovascular events in patients with RA as a potential predictive biomarker.

Conclusion

In conclusion, in our preliminary study of RA, PsA and NIA, we found some differences in protein expression pattern between inflammatory and non-inflammatory rheumatic conditions. Regarding distinction between RA and PsA, four proteins were down-regulated in PsA and up-regulated in RA as compared to NIA (SERPIN A11 – Q86U17, complement factor H-related protein 5 – Q9BXR6, cartilage acidic protein 1 – Q9NQ79 and coagulation factor IX – P00749). These results can be further used as a starting point to explore the potential of these plasma proteins as potential biomarkers for RA and PsA. A larger study is needed to refine our findings regarding the level of inflammation associated with the expression of specific proteins.

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