Metabolic and Inflammatory Proteins Differentially Expressed in Platelets from Unprovoked Deep Vein Thrombosis Patients

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Abstract

Deep vein thrombosis (DVT) is multi-causal disease associated to high morbidity and mortality due to complications, especially from pulmonary embolism. New factors of relevance on the DVT pathophysiology enhance our understanding of disease mechanisms and can be translated into improved management of patients, prevention of recurrence and development of new therapies. In this context, the precise role of platelets in the pathogenesis of DVT is not completely understood. Our objectives were to acquire and to analyze the whole platelet protein profile of samples from 3 DVT patients and to compare them to results obtained from 1 sibling and 1 neighbor from each patient (in order to minimize genetic and environmental interferences). These patients presented unprovoked and recurrent episodes of proximal DVT as well as a family history of DVT. Platelets were washed, lysed, and the proteins were hydrolyzed by trypsin. Peptides were first separated by HPLC and peptide fractions were further detected by LC-MS/MS. Five proteins were present on patients and absent in all the controls: Apolipoprotein A1 Binding-Protein, Coatamer (zeta1 sub-unit), 17β-hydroxysteroid dehydrogenase type XI, Leukotriene A-4 Hydrolase and Sorbitol Dehydrogenase. The analysis identified proteins that currently are not related to the pathophysiology of DVT, and the persistence of these inflammatory and lipid transportation-related proteins emphasize the relevance of these phenomena on DVT.

Keywords: Platelets; Deep vein thrombosis; Inflammation; Lipid transportation; Metabolism

Introduction

Thrombosis is a multi-causal pathological process resulting from the activation and propagation of the hemostatic response. Venous thromboembolism (VTE) is considered a common disease, and its incidence ranges from 1 to 3 cases per 1000 individuals [1,2]. In addition, nearly 25% develop recurrent VTE within 10 years. Several hereditary and acquired risk factors have been identified in the last two decades, but about 25% of patients present unprovoked deep vein thrombosis (DVT), without known risk factor [3]. Additionally, patients with the same genetic alteration can present diverse clinical characteristics, suggesting a possible interaction with other factors on DVT triggering. So, the identification of pathways involved on the pathophysiology of DVT would have great importance in clinical practice.

Traditional coagulation tests, especially clot-based assays, are useful for describing major abnormalities on haemostatic response, but fail to assess thrombotic risk in the healthy population. Recent evidence also attests that the analysis of a vast array of genes can only explain a part of the individual thrombotic risk [4]. Thus, proteomic analysis may hold promise for characterizing or understanding biological pathways and pathophysiological interactions. To date, abnormal protein expression profiles were identified by proteome approaches in patients with increased risk of DVT. Our group previously performed a plasma proteomic profile with the same individuals included in this study, and verified that inflammatory, immune and lipid transportation proteins were differentially expressed [5]. Other recent findings included the identification of one antithrombin variant (P80S) [6], which cannot be efficiently secreted from the hepatocyte, associated to loss of function. Gelfi et al. [7] verified increased level of glycosylation in carriers of the G20210A prothrombin mutation, which confer it a greater stability. Another study performed on low molecular weight (500–20,000 Da) proteins noticed that they could be useful in differentiating members of a type I protein C deficiency family who has suffered thrombotic events before the age of 40 years from disease-free family members [8].

Platelets have a multitude of physiological functions, as the control of haemostasis, the regulation of the vascular tone, the interaction with leukocytes in inflammatory reactions, vascular repair and the stimulation of cellular proliferation by the supply of growth factors [9]. Studies in platelet cytosolic proteome identified glycoproteins and protein defects in patients with hereditary hemorrhagic diseases [10] as well as after stimuli [11-15]. Microparticles from DVT patients showed increased expressed (galectin-3 binding protein and alpha-2 macroglobulin) and depleted (fibrinogen beta and gamma chain precursors) proteins [16].

In this scenario, our objective was to acquire and to analyze the whole platelet protein profile of samples from 3 high-selected patients with unprovoked and recurrent DVT, and to compare them to others obtained from healthy siblings and neighbors.

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Patients and Methods

Patients were recruited from the Outpatient Clinic of Hemoctetro de Campinas/UNICAMP between February 2009 and September 2009. We selected 3 patients with recurrence of DVT episodes confirmed by Doppler ultrasonography, at least 6 months after withdrawal of warfarin therapy. Patients older than 50, with acquired or a known hereditary thrombophilia, cancer, myeloproliferative syndrome, liver or renal disease were excluded. In order to minimize the environmental and genetic influence in protein expression, two control groups composed by siblings and individuals originated from the same geographic area were included. These neighbors had no personal or familial history of DVT, and were matched by gender, age (+/- 5 years), predominant ethnic origin and tobaccoism. None of the patients and controls had taken antplatelet or anti-inflammatory drugs for 15 days before the blood collection. This study was performed in accordance with the Declaration of Helsinki and was approved by our local medical ethics committee. All patients and controls signed a written informed consent.

Sample collection

Blood (24 mL) was drawn from the antecubital vein. The first sample was collected into a vacuum tube (Greiner Bio-One, Kremnnsunter, Austria) containing EDTA and was directed to a blood count. The second sample was collected with a wide-bore (16-gauge) needle with syringe containing acid-citrate-dextrose anticoagulant solution (pH 4.5, 1 mL of ACD for each 6 mL of blood) as previously described by Cazenave et al. [17]. Platelets were isolated immediately after blood collection and submitted to resting periods at 37°C for 15 min between centrifugations in order to revert initial activation processes. Platelets were pelleted from the platelet-rich plasma and washed twice in Tyrode’s albumin solution (145 mM NaCl, 5 mM KCl, 1 mM MgCl2, 10 mM HEPES, 10 mM glucose, pH 7.4) supplemented with 10 U/mL of heparin and 0.5 µM prostacyclin (PGE1) (Sigma-Aldrich, St. Louis, MO, EUA) followed by centrifugation at 1900 x g for 8 min. The cell pellet was collected into a vacuum tube (Greiner Bio-One, Kremsmunster, Austria) and the platelets were finally pelleted by centrifugation, frozen in liquid nitrogen and stored at -80°C.

Protein extraction and digestion

The protein extraction and digestion were performed according to Wojcechowskyj et al. [18]. Briefly, platelets were thawed on ice and the lysed (SDS 0.3%, 20 mM DTT in a tris buffer) in presence of protease inhibitor (P8465, Sigma-Aldrich, St. Louis, MO, EUA). After boiling for 5 min, nucleic acids were removed with Benzonase 10% (Novagen®, Madison, WI – EUA) and the proteins were alkylated with iodoacetamide (final concentration 50 mM). Proteins were precipitated with acetone and were immediately digested with trypsin (10ng/µL Porcine Trypsin - Promega, Madison, WI, USA). Salts were removed applying Sep-Pak columns (C18, Waters®, Milford, MA, USA) according to the manufacturers instructions.

Hydrophilic Interaction Chromatography (HILIC) and mass spectrometric analysis

The HILIC was performed according to McNulty and Annan [19]. The peptides were fractionated by the TSking Amide-80 column (21487, 1 mm x 25 cm, 5 Å, TOSOH Bioscience®, Tokyo, Japan) connected to the UPLC (Shimadzu Scientific®, Duisburg, German) after three consecutive runnings of 1 µg/µL of the MassPREP BSA Digestion Standard (Waters®). The variability observed on the obtained chromatograms was visually compared to others obtained from three runs of each individual included in a group of analyzes (one patient, one sibling and one neighbor). As a similar variability was obtained in these comparisons, the subsequent runs were considered accepted.

The gradual release of the peptides from the column occurred according to their affinity to a hydrophilic (A: 0.1% TFA) and a hydrophobic (B: 98% ACN/0.1% TFA) buffers. The fractions were collected in 8 intervals of 10 minutes each, from t=5 to t=85 min, and frozen in liquid nitrogen. They were then lyophilized, resuspended in 40 µL of 0.1% formic acid and directed to the hybrid mass spectrometer LTQ-Orbitrap (Thermo Fisher Scientific, San Jose, CA, USA) and auto-sampler coupled with a pump NanoLC (Eksigent Technologies, Livermore, CA, USA). A second reverse phase chromatography was performed on a nanocapillary column: 75 mm (L.D.)×20 cm ProteoPep (New Objective®, Woburn, MA, USA). The buffer A consisted of 1% methanol/0.1% formic acid and the buffer B consisted of 1% methanol/0.1% formic acid/79% ACN and the runs comprised a 15 min. sample load at 3% B, and a 90 min. linear gradient from 5 to 45% B. The mass spectrometer was set to repetitively scan m/z from 300 to 1800 (R=100,000 for LTQ-Orbitrap) followed by data-dependent MS/MS scans on the six or ten most abundant ions, with a minimum signal of 1000, isolation width of 2.0, normalized collision energy of 28, and waveform injection and dynamic exclusion enabled. FTMS full scan AGC target value was 1×, while MSn AGC was 5×, respectively. FTMS full scan maximum fill time was 300 ms, while ion trap MSn fill time was 50 ms; microscans were set at one. FT preview mode, charge state screening, and monoisotopic precursor selection were all enabled with rejection of unassigned and 1+ charge states. The DTA files extracted from the RAW data files were processed into the Sorcerer-SEQUENT (ver. 4.0.3, rev 11; SagenReseach, San Jose, CA, USA). The search was performed against the REV US_HUMAN_TS_2MC_090324 database, with a parent tolerance of 1.0 Da, a peptide mass tolerance of 50 ppm and one trypsin missed cleavage. Iodoacetamide derivative of cysteine and oxidation of methionine were specified as fixed and variable modifications, respectively. The potential sequence-to-spectrum peptide assignments generated was loaded into Scaffold 3 (Scaffold 3_00_3, Proteome Software Inc. Portland, OR, USA) to validate MS/MS peptide and protein identifications and applied the xcorr cutoffs (+1>1.8, +2>2.5 and +3>3.5). A peptide was considered as unique when it differed in at least 1 amino acid residue; modified peptides, including N- or C-terminal elongation (i.e. missed cleavages) were also considered as unique while different charge states of the same peptide and modifications were not counted as unique. The Peptide and Protein Prophet algorithms calculated peptide and protein probabilities respectively. The variability was analyzed by two consecutive injections of each individual of one group of analyzes (one patient, one sibling and one neighbor), and as similar number of proteins was obtained, the subsequent injections were performed as unique experiments.

Results

According to the inclusion and exclusion criteria, from the 30 pre-selected subjects, 3 were selected due their unprovoked and recurrent DVT, positive family history and availability of appropriate controls. They consisted of one male and two female individuals, two Caucasian and one Afro-descendent (see demographic data on Table 1). None of the patients reported use of tobacco, diabetes, cancer, hypothyroidism, renal or liver disease, but one of them was under treatment for arterial hypertension with amloidipine besylate and atenolol. All patients presented proximal DVT and the time between the last DVT event...
Platelet counts varied from 263 to 312, 185 to 365 and from 230 to 466×1000/µL between patients, siblings and neighbors respectively. In the last step of washing, the contamination with leukocytes and erythrocytes was estimated to be ≤ 0.2% of platelet population [leukocytes med=0.09% (0-0.2%); erythrocytes med=0.03 (0-0.007%)]. In the end of the analysis, proteins typically associated with RBCs (such as α and β-globin or spectrin) were not detected, confirming that the contamination with these cells in the isolated platelets was minimal.

Applying the HILIC technology, the peptides were successfully fractionated, enhancing the identification of the proteins after the mass spectrometry analysis. Figure 1 illustrates the chromatograms showing the platelet peptides from a DVT patient (Figure 1A), his sibling (Figure 1B) and his neighbor (Figure 1C). Figure 1D shows the superposition of the chromatograms, illustrating the interindividual differences among the analyzed group.

### Mass spectrometry

The methodological variability was analyzed over the proteins identification from all the subjects included in this study. The number of proteins identified is shown on Figure 2, and was very similar in each set of analysis. The variability observed was considered acceptable for the methodologies applied.

The presence and absence of the proteins was evaluated by two different ways: proteins present in all the patients and absent in all the controls and vice-versa. On total, 5 proteins were present in all the patients and absent in all the controls. They were: ζ 1 sub-unit of coatomer (COPZ1), apolipoprotein a1 binding protein (APOA1BP), leukotriene A4 hydrolase (LTA4H); 17β hydroxysteroid dehydrogenase type XI (HSD17B11) and sorbitol dehydrogenase (SORD). When we inverted the analysis, looking for proteins absent on the patients and present on the controls, none of the proteins remained constant in all the evaluated individuals.

### Discussion

Platelets are important mediators of haemostasis and thrombin generation, and there is no doubt about their relevance in the pathophysiology of VTE. In this study, we employed a proteomic approach to identify platelets proteins from unprovoked and recurrent DVT patients and to compare to profiles obtained from their siblings and neighbors.

The platelet proteome is subject to rapid changes in response to external signals, giving rise to potentially large within and between subject variation [20]. In this study, the methodology applied to isolate platelets with minimum activation and contamination with other cells and plasma minimized the impact of the cells to the tube on the collection with syringe. The first tube was discarded due thrombin generated by the vessel wall lesion [17]. The anticoagulant ACD better prevents the platelet spontaneous activation [21], and the wash buffers with glucose, physiologic pH and PGE1 inhibited the platelet function by decreasing platelet fibrinogen receptors expression and P-selectin [22]. Briefly, PGE1 is produced by the endothelial cells and blocks the calcium release from the dense tubular system and also the triphosphate inositol and several kinases release, preventing the platelet aggregation [23]. The washes intercalated with incubations at 37°C could revert initial activation processes. Another interesting point was the inclusions of two different controls in order minimize the genetic and environmental influences over the proteins expression, which increased the reliability and the stringency over the results as well.

We identified 5 proteins that were only present on patients: the apolipoprotein A1 binding-protein, the ζ 1 sub-unit of coatomer, the 17β hydroxysteroid dehydrogenase type XI, the leukotriene A-4 hydrolase and the sorbitol dehydrogenase. All those proteins are involved on lipid transportation, metabolism and inflammation. Interestingly, LTA4H and SORD had never been described in platelets.

Platelets enhance vascular injury through thrombotic mechanisms, but also appear to help orchestrate pathologic immune responses and are pivotal players in facilitating leukocyte recruitment to vulnerable tissue [24].

Leukotriene A4 hydrolase (LTA4H) is a proinflammatory enzyme that generates the mediator leukotriene B4. Qiu and colleagues reported increased mRNA and protein levels of LTA4H in 72 human carotid atherosclerotic plaques compared to 6 controls. There was a correlation between increased levels of LTA4H mRNA and symptoms of plaque instability, which promotes activated neutrophils to produce LTB4 and inducing the myeloperoxidase activity [25]. It generates potent oxidants and renders them proinflammatory [26-28], also inactivating protease inhibitors and consuming nitric oxide, all of which escalate the inflammatory response [29]. Helgadottir et al. [30] showed that a haplotype spanning the LTA4H gene confers higher risk of myocardial infarction in an Icelandic cohort. Neutrophils from myocardial infarction cases with the at-risk haplotype resulted in more LTB4 than controls. As the inflammation process is a common event to the arterial and venous disease, the presence of LTA4H only on platelets from DVT patients corroborates the role of this protein in this process. These findings are in accordance with our previous proteomic study on plasma samples from these same individuals, which showed a different expression of proteins directly or indirectly involved with inflammation, like serum amyloid A, C1 inter-alpha-trypsin inhibitor, heavy chain H inhibitor and isoform 2 of inter-alpha-trypsin inhibitor heavy chain H4, alpha-2-HS-glycoprotein [5]. The presence of another inflammatory protein on the platelets proteomic analysis can indicate a sustained inflammatory status on those DVT patients, which could enhance not only the platelet activation, but also could retro-feed the process stimulating the neutrophils and endothelial cells and their interactions.

The 17β hydroxysteroid dehydrogenase type XI (HSD17B11) converts androstan-3-alpha,17-beta-diol to androsterone in vitro [31], and participate in androgen metabolism during steroidogenesis. The involvement of this protein on the coagulation disorders is presently contradictory. Estrogen is known to have multiple protective effects on the cardiovascular system [32]. Wang and Abdel-Rahman [33] also showed that HSD17B11 deficiency leads to a decrease in total cardiac NOS activity due to changes in NOS3 activity. Estrogen also modulates the activity of the mitogen-activated protein kinase (MAPK) pathways.

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**Table 1:** Demographic data from patients with Deep Vein Thrombosis (DVT), siblings and neighbors.

<table>
<thead>
<tr>
<th></th>
<th>N of males/females</th>
<th>Mean age: min-max (years)</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Patients</td>
<td>1 / 2</td>
<td>46.33 ; 44-50</td>
<td></td>
</tr>
<tr>
<td>Siblings</td>
<td>1 / 2</td>
<td>49.67 ; 41-56</td>
<td>0.7</td>
</tr>
<tr>
<td>Neighbors</td>
<td>1 / 2</td>
<td>46.00 ; 40-50</td>
<td>0.83</td>
</tr>
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Figure 1: Chromatograms obtained on the peptides fractionation by reverse phase applying the UPLC in platelets from a DVT patient (A), his sibling (B) and his neighbor (C). (D) Shows the superposition of the chromatograms, illustrating their interindividual differences.

Figure 2: Number of proteins identified after mass spectrometry analysis of platelets peptides from 3 unprovoked DVT patients, their 3 siblings and 3 matched neighbors. The black line shows the number of common proteins identified between the analyzed groups.
in cardiac myocytes [34,35]. However, contraceptive use and the post-menopausal hormone therapy are established risk factors to DVT [36]. HSD17B11 repress the protein S alpha gene and the production of its mRNA in HepG2 and ER α cells. HSD17B11 down-regulation on the hormone replacement would reduce the tissue cortisol production, allowing the progression of both vascular and pulmonary inflammation [37]. Our results regarding the presence of HSD17B11 only in platelets from patients favored a deleterious effect of this protein on the DVT pathophysiology.

The COPZ sub-unit of the coatamer (COPZ1) is a 700 kDa cytosolic protein complex consisting of seven equimolar subunits (α-, β-, β′-, γ-, δ-, ε- and ζ). The coatamer complex and the ADP-riboseylation factor I are the main components of coat protein complex I (COP1) [38]. The perturbation in COPI increased the storage of triglycerides by decreasing the lipolysis rate, and it was demonstrated a positive regulation of lipolysis by the coatamer retrograde-vesicle trafficking pathway [39]. Dekroon and Armatti [40] verified that apolipoprotein E synthesis depends on the COPZ1 complex.

Apolipoprotein A-I Binding Protein (APOA1BP) is directly bound to apolipoprotein A1 (APOA1) [41], which is considered anti-atherogenic by being a part of the chylomicrons and HDL. In Budd-Chiari syndrome, the patients showed a significant decrease of APOA1 plasma levels [42]. Fasting levels of FVIIa and FVII-Ag correlate to serum triglycerides and APOA1 [43]. Fatty meals might lead to endothelial dysfunction, hypercoagulability and platelet hyperactivity, which predispose to acute episodes of thrombosis [44,45]. Controversial results were found by a longitudinal study in 114 rheumatoid arthritis patients, which demonstrated an independent negative prediction of carotid intima-media thickness by APOA1, but a positive by APOB/APOA1 ratio. This complex ratio is also considered strongly predictive for ischemic stroke in elderly subjects [46].

Our hypothesis is that the APOA1 could be a protective protein released by the organism in critical situations in order to minimize the damage, what is corroborated by Jin et al. [47], who verified increased plasma HDL levels in postmenopausal women under estrogen therapy, reducing the cardiovascular risk. The work of Jha et al. [48] suggested an enzymatic function for APOA1BP to include a nucleotide-containing substrate during capacitation at the level of cholesterol efflux. APOA1BP showed a concentration-dependent release into the media in cells derived from kidney proximal tubules after APOA1 or HDL stimulation, implicating a role on the renal tubular degradation or resorption of APOA1. This found suggested that the ostensive presence of the APOA1BP would be a consequence of the APOA1 increased levels. Interestingly, in our study the APOA1 levels in platelets samples were similar between patients the controls.

The aldose reductase (AR) promotes the reduction of glucose to sorbitol, which is oxidized by sorbitol dehydrogenase (SORD) to fructose. Recently, the sorbitol via had been implicated in osmotic and oxidative stress [49]. The inhibition of AR and SORD in cerebral ischemic injury [49], myocardial infarction [50] and with a pharmacological inhibitor (Fidarestat) protected animals from severe neurological deficits and large infarct. SORD could enhance oxidative stress and in turn contribute to oxygen privation and subcutaneous tissue. Common factors of arterial and venous disease could indicate a role of stress oxidative also on DVT.

Our study has some limitations. The number of patients included in this study is relatively small to make definitive conclusions. However, our idea was to perform a pilot and exploratory study comparing protein profiles obtained in homogeneous high-selected patients with unprovoked and recurrent DVT episodes. Other point was the election of one group of patients which were not exclusively composed by one gender or ethnic origin. We recognize that the inclusion of this diversity of individuals could dilute some important differences related to these aspects, but our objective was to sensibilize the samples for detection of alterations strongly associated to the clinic phenomenon and not to other characteristic not related to DVT. In addition, platelets were isolated between 1 and 4 years after the event and their proteome examined at a single time-point.

Summarizing, a diverse group of proteins were differentially expressed in platelets from recurrent DVT patients in comparison to siblings and neighbors, and they were mostly associated with an inflammatory status, metabolic and lipid transportation alterations. The persistence of these inflammatory and lipid transportation-related proteins in both proteomic studies performed by our group as well as in the recent literature emphasize the relevance of these phenomena on DVT.

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