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Proteomics of Microparticles after Deep Venous Thrombosis

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Abstract

Background—Microparticles (MP) are submicron size membrane vesicles released from activated cells that are associated with thrombosis and inflammation. MP present diverse biological expressions that may be linked to a unique subset of proteins derived from their origin cells.

Methods—To identify these proteins, plasma samples were taken from 9 patients with deep venous thrombosis (DVT) documented by duplex ultrasound, 9 with leg pain but negative for DVT by duplex, and 6 healthy controls without a history of thrombosis, for fold variation. MP were extracted from platelet-poor plasma, digested separately with trypsin and tagged using iTRAQ reagents. The digests were subjected to 2-D LC separation followed by MALDI tandem mass spectrometry. Peak lists were generated and searched against all human sequences. For protein identification, a minimum of two peptides at 95% confidence was required. Later, iTRAQ ratios were generated comparing relative protein levels of DVT patients to baseline. The proteomic analysis was performed twice for each blood sample. Proteins were considered elevated or depressed if the iTRAQ ratio (R) deviated by 20% change from normal and a p-value less than 0.05.

Results—Two proteins (Galectin-3 Binding Protein, [Gal3BP], R=1.76 and Alpha-2 macroglobulin [A2M] R=1.57) were differentially expressed on DVT patients. Nine proteins were depleted including fibrinogen beta and gamma chain precursors (R=0.65).

Conclusions—These proteins influence thrombosis through inflammation, cell shedding, inhibition of fibrinolysis and hemostatic plug formation. Further studies are needed to confirm the mechanistic role of these proteins in the pathogenesis of venous thrombosis in humans.

Keywords

Microparticles; Microparticle proteins; Proteomics; Venous Thrombosis

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Conflict of interest

None.

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Introduction

Venous thromboembolism (VTE) remains a significant health problem in the United States with an estimated 900,000 cases of deep venous thrombosis and pulmonary embolism yearly¹. The pathophysiology of venous thrombosis has traditionally included stasis of the blood, hypercoagulability and changes in the vein wall (Virchow's triad).

In the 1970's Gwendylen Stewart suggested a relationship between thrombosis and inflammation². Recent studies have investigated the role of procoagulant microparticle (MP) formation in the inflammatory component of venous thrombosis³.

MP are small fragments of cell membrane, present in plasma and shed from different cells and elements such as platelets, leukocytes and endothelial cells. Some MP are rich in tissue factor (TF) and facilitate and amplify coagulation in the presence of thrombus. In addition, MP promotes an interface between inflammation and thrombosis. These small structures are fragments of phospholipid membrane released from different elements and cells in activation and apoptosis; in addition, recent evidence suggests MP carry RNA⁴ and DNA⁵. MP may concentrate certain proteins, and have been characterized by evaluating platelet-derived and plasma-derived MP obtained from healthy individuals.^{6, 7} Fibrinogen was enriched on MP in a nonhuman primate model of venous thrombosis^{8, 9}. However, there has been no description of MP protein up-regulation or depletion on human MP in patients with venous thrombosis. Therefore, the purpose of this study was to use proteomic evaluation to identify these proteins, helping to understand the role of MP in human venous thrombogenesis.

Methods

Twenty four (24) subjects make up this study, 9 patients with DVT documented by duplex ultrasound, 9 patients with leg pain but negative for DVT by duplex, and 6 healthy controls with no history of venous thrombosis. Controls were used for fold change; comparisons were made between patients with positive *versus* negative DVT confirmed by duplex scan, adjusted by controls. Three experiments with 8 patients each (3 DVT positive, 3 DVT negative and 2 controls per experiment, with a total of 24 subjects were performed). At the time of diagnosis (patients) or donation (controls), subjects had an 8.5-mL tube of whole blood drawn into 10% acid citrate dextrose (ACD) by butterfly antecubital stick. Platelet poor plasma (PPP) was obtained by centrifuging blood at 1,500xg and room temperature for 25 min, transferring the plasma to another tube and centrifuging it once more for 2 min at 15,000xg, to remove contaminating cells from the plasma. PFP (4-mL) obtained from each subject was stored in 1-mL aliquots at -70°C for 12 and 24 months. We have performed a pilot study in our methods to compare frozen from fresh samples variability. Testing blood from 4 healthy donors, no significant differences regarding plasma total proteins concentrations, total MP, platelet and monocyte derived MP counts between frozen and fresh samples were observed.

For proteomic evaluation, PPP was thawed and 400µL was spun down in 1-mL of HEPES buffer [10 mM Hepes/5 mM KCl/1 mM MgCl₂/136 mM NaCl₂ (pH 7.4)] for 2 h at 4°C, 200,000 × g. Supernatant was removed and MP pellet was resuspended in 400-µl of 0.25M KBr and incubated on ice for 20 min. Samples were then spun down for 2 h at 4°C, 200,000 g. Supernatant was removed and pellet air dried before being resuspended in 250-µl of 1X PBS. The previous steps involving suspension in KBr followed by centrifugation were performed in all experiments to remove soluble serum proteins. Protein concentration was determined using a standard colorimetric BCA total protein assay (Pierce, Rockford, IL).

Protein Isobaric Labeling with iTRAQ Reagents

MP from 200 μ L PPP were suspended in 20- μ L 0.5M triethylammonium bicarbonate (TEAB) and 0.1% sodium dodecyl sulfate (SDS). For four-plex isobaric labeling, separate aliquots of proteins were treated in parallel, essentially as described by Ross et al. Stock reagents and buffer (TEAB, SDS, Tris (2-carboxyethyl)phosphine (TCEP), S-methyl methanethiosulfonate (MMTS), and the four isobaric tagging reagents) were obtained in kit form (Applied Biosystems, Foster City, CA). Protein (51.5 μ g) was reduced with 2.5 mM TCEP (60°C for 1 h) and cysteine residues blocked with 10 mM MMTS (room temperature for 15 min). Protein was digested with trypsin (porcine modified, Promega; 1:20, w/w) for 20 h. Isobaric tagging iTRAQ reagent (1 unit in 70- μ L ethanol) was added directly to the protein digest (70% ethanol final) and the mixture incubated at room temperature for 1 h. The reaction was quenched by addition of 9 volumes 0.1% trifluoroacetic acid (TFA) in water. The reaction mixtures were combined and stored at 4°C.

SCX Peptide Fractionation

For the first dimension of the two-dimension chromatographic separation, an aliquot of the four-plex peptide mixture (200 μ g) was applied to a sulfoethyl aspartamide SCX spin column (SEM HIL-SCX, PolyLC, The Nest Group, Inc. Southboro, MA) equilibrated with 10 mM KH_2PO_4 , pH 4.5, 20% CH_3CN . Excess reagent was washed from the column with 800- μ L equilibration buffer. Peptides were eluted using 50 μ L volumes of KCl in a stepwise gradient from 25–500 mM KCl in equilibration buffer (25, 50, 75, 100, 150, 225, 350, and 500 mM KCl). Fractions were dried in a vacuum centrifuge.

Reversed-Phase Liquid Chromatography

For the second dimension separation, peptides in SCX fractions were separated by C_{18} nano LC using an 1100 Series nano HPLC equipped with μ WPS autosampler, 2/10 microvalve, MWD UV detector (214 nm) and Micro-FC fraction collector/spotter (Agilent). Each SCX salt step was reconstituted with 43- μ L 0.1% TFA, v/v in water and 40- μ L injected. Sample was injected onto a C_{18} cartridge (Zorbax300SB, 5 μ m, 5 $\times$ 0.3 mm; Agilent), desalted with solvent C ($\text{CH}_3\text{CN}:\text{H}_2\text{O}:\text{TFA}$, 5:95:0.1) at 20- μ L/min for 9 min with the effluent discarded. The enrichment cartridge was placed ahead of a C_{18} column (Zorbax300SB, 3.5 μ m, 150 $\times$ 0.1 mm; Agilent) equilibrated with solvent A ($\text{H}_2\text{O}:\text{TFA}$, 99.9:0.1). Peptides were eluted with a gradient of solvent B ($\text{CH}_3\text{CN}:\text{H}_2\text{O}:\text{TFA}$, 90:10:0.1) from 6.5% B to 50% B over 90 min at a flow rate of 0.4 μ L/min. Column effluent was mixed (micro Tee, Agilent) with matrix (2 mg/ml α -cyano 4-hydroxy cinnamic acid (CHCA in CH_3OH : isopropanol: $\text{CH}_3\text{CN}:\text{H}_2\text{O}$: acetic acid (12:33.3:52:36:0.7) containing 10 mM ammonium phosphate) delivered with a PHD2000 infusion pump (Harvard Apparatus) at 0.9 μ L/min. Fractions were spotted at 24 s intervals (192 fractions/LC run) onto large format stainless steel MALDI targets (Applied Biosystems).

Mass Spectrometry

Mass spectra were acquired on an Applied Biosystems 4800 MALDI TOF/TOF Analyzer (TOF/TOF). MS spectra from 800–3500 Da were acquired for each fraction from 750 laser shots of a 200 Hz YAG laser operated in the 3rd harmonic (355 nm). The TOF/TOF was operated in positive ion reflectron mode. Seven point Gaussian smoothing was applied to spectra and a S/N of 30 filter applied for peak picking. Calibration was done using default mode. Plate calibrants were glufib (m/z 1714.787), ACTH (m/z 2753.419), angiotensin (m/z 1440.790), bradykinin (m/z 1048.578), and ACTH 1–17 (m/z 2107.197). The twelve most intense peaks in each MS spectrum were selected for MS/MS analysis. MSMS spectra were acquired from 1500–400 laser shots using “quality dependent” mode (6 peaks at S/N 60); fragment peak picking used S/N 40. MSMS calibration was done with fragments of glufib (m/z 430.242, 684.346, 1056.475 and 1441.634). Fragmentation of the labeled peptides was

induced by the use of atmosphere as a collision gas with a pressure of approximately 6×10^{-7} torr and collision energy of 2kV.

Peptide identifications were performed using GPS Explorer (v3.6, Applied Biosystems) which acts as a front end for the Mascot search engine (v2.1 MatrixScience, London UK). Each MS/MS spectrum was searched against NCBI mammal (Feb. 2, 2007). Trypsin specificity with one missed cleavage was selected. S-mercaptopmethionine and the N-terminal and lysine iTRAQ labels were selected as fixed modifications. Oxidized methionine was considered as a variable modification. The precursor tolerance and MS/MS fragment tolerances were ± 0.7 and ± 0.3 Da, respectively. Individual peptide identifications were grouped into protein identifications using GPS Explorer and assigned a total ion C.I.%. Only the peptides with C.I. % at 95% or above for any MS/MS spectrum were retained for further analysis.

Statistical Analysis

The data were processed as follows. The raw peak areas for each experiment were normalized following the methodology in Keshamouni *et al.*,¹⁰ using a quantile method. Subsequently, the desired ratios of DVT versus controls for each experiment were obtained using the random effects ANOVA model introduced in Keshamouni *et al.*¹⁰ and Jagtap *et al.*¹¹ This model considers the hierarchical structure in the data, namely that every protein is comprised of several peptides and the expression level of each peptide may be measured by multiple spectra. The outcome of this model is a ratio (R) of the treatment effect (2-day expression level) over the control effect (baseline expression level), along with its standard error, which allows us to obtain the p-value for testing the hypothesis R is in the range (0.8–1.2) –no change, versus the alternative one that R is outside that range. The choice of this range is supported by experience with previous experiments, where such ratios usually do not indicate biological activity. Proteins with p-values smaller than 0.05 that rejected the null hypothesis of no change were retained for further processing.

The p-values were adjusted since multiple experiments (3 experiments with 8 samples each: 3 DVT positives, 3 DVT negatives and 2 controls [total n=24]) were performed and some proteins appeared significant in several of them. A false discovery rate¹² approach was used to appropriately adjust the p-values, in order to make the appropriate differential expression calls.

All patients signed the informed consent previously to any study procedure. The protocol was approved by the institution IRB.

Results

Protein identifications were based on two peptides with 95% confidence. Of the 151 proteins identified, 35 proteins displayed a greater than 20% enrichment or depletion on MP, in 2 out of 3 experiments, as listed in table 1. Special significance was given to proteins with their level of expression being regulated appearing in 3 out of 3 experiments. Eleven proteins fit this criterion and are listed in Table 2. Among these proteins, two were enriched in MP from DVT patients; the first class of proteins represented was Galectin-3 binding protein (Gal3BP), iTRAQ ratio R=1.76. The second class, Alpha-2 Macroglobulin (A2M) was also enriched (R=1.52). All 151 proteins identified are shown on table 3.

Nine proteins were depleted in MP of DVT patients; among them, fibrinogen beta chain precursor (R=0.65) and fibrinogen gamma chain precursor, (R=0.64). Other polypeptides, not related to thrombosis or inflammation, were also depleted as shown in table 2. All of these proteins were depleted on DVT patients compared to negatives as indicated by iTRAQ ratios of less than 0.8. Complementary information (DVT positive and DVT negative distribution, D-dimer levels, MP count) is shown on table 4.

Discussion

Thrombosis and inflammation are inter-related processes. At the time of thrombus formation, MP are recruited to the thrombus site, playing a role in thrombus modulation.

MP are submicron vesicles shed from plasma membranes, in response to cell activation, injury and/or apoptosis. Recent investigations suggest that MP, which are prothrombotic in part by virtue of carrying TF,^{13–16} are important in early venous thrombogenesis.¹⁷ MP are recruited to the area of thrombosis,¹⁸ where they amplify coagulation via TF and factor VIIa.^{13, 17–19} Co-localization of fibrin, platelets, and leukocytes in the developing thrombus^{13, 20} and the leukocyte-platelet interactions generating TF²¹ support the central role of inflammation in thrombogenesis²², with MP interacting both in coagulation and inflammation processes. MP are also found in healthy individuals²³, as well as pathological conditions such as severe sepsis, where their reduction was found to correlate with organ dysfunction and mortality.²⁴ The current study evaluated MP proteins with proteomics analysis in human venous thrombosis. Since MP present diverse biological expressions that may be linked to a unique subset of proteins, derived from their origin cells, the assumption is that those proteins are crucial for influencing thrombogenesis and are regulated during the course of venous thrombosis. Thus, identifying and understanding the nature of these proteins can serve to provide valuable insight into the mechanistic role of MP in human venous thrombogenesis.

Enriched Proteins: Gal3BP and A2M

Gal3BP precursor is a polypeptide from the lectin family that promotes integrin-mediated cell adhesion²⁵. In addition, it plays a key role in human platelet activation and function, synergizes with ADP or thrombin to induce platelet aggregation and ATP release, induces up-regulation of P-selectin, induces GPIIb expression, promotes shedding of MPs, and favors the generation of leukocyte-platelet aggregates²⁶. Those effects of Gal3BP may play an important role in thrombosis and inflammation, potentially amplifying the thrombus progression. Gal3BP was enriched in MP of all DVT patients, leading to the idea that this protein is over-expressed during thrombus formation process. Previous studies have demonstrated that P-selectin inhibition decreases thrombogenesis²⁷. An inhibition of Gal3BP would promote an upstream inhibition of P-selectin and even a decrease in MP generation, decreasing thrombus propagation.

On the other hand, A2M plays an important role in thrombogenesis and fibrinolysis. It functions as an inhibitor of fibrinolysis by inhibiting plasmin and kallikrein, and as an inhibitor of coagulation by inhibiting thrombin. In all DVT patients A2M was enriched. MP, by virtue of having this inhibitor, may play a role in thrombus formation (by its inhibition of plasmin) facilitating thrombus progression.

Proteins Depleted in MP of DVT Patients

Nine proteins were observed to be depleted in MP from DVT patients compared to controls. Seven of them, Serpin1 Isoform 1 of Alpha-1-antitrypsin precursor, Histidine-rich glycoprotein precursor, Hemopexin precursor, Transferrin variant serotransferrin precursor, Fibrinogen, Serum albumin precursor and Rheumatoid factor RF-IP16, play no known major role in inflammation or thrombosis.

Fibrinogen is a six-chain protein precursor to the clot structural protein, fibrin and dimer of α , β , and γ polypeptides.^{28–29} Proteomic analysis detected 2 of these polypeptides (Fibrinogen beta chain precursor and Isoform Gamma-B of Fibrinogen gamma chain precursor) on MP, both depleted on MP from DVT patients. The presence of fibrinogen suggests a number of potential mechanisms regarding the role of MP in the thrombotic process. Interestingly, in our previous study of experimental DVT in primates, fibrinogen was enriched in early stages of

thrombosis (day 2)⁸. In spite of having all samples collected before the initiation of DVT treatment, the time point of blood collection in human samples cannot be precise, as it was in our controlled experimental primate model, where samples were collected precisely 48 hours after thrombus initiation. Many patients do not present immediately upon the development of leg symptoms and we thus have no way to know the precise timing of thrombus initiation. The down-regulation of fibrinogen in this study may be due to its consumption (fibrinogen taken into the thrombus and consumed).

Unexpectedly, a number of proteins such as tissue factor (TF), P-selectin, and P-selectin glycoprotein ligand-1 (PSGL-1) were not observed on MP using proteomics analysis, in both experiments (this human DVT MP analysis and our previous experimental primate DVT model study⁸). This is interesting since it was reported that the amount of TF activity on MP correlate with the presence or absence of experimentally induced venous thrombosis^{15, 16, 30, 31} and P-selectin and PSGL-1 has been found critical in venous thrombogenesis. Potential reasons for this absence include the saturation of these proteins by their receptors, or glycosylation. Highly glycosylated proteins might not be detected by proteomic analysis, and PSGL-1 as a receptor for P-selectin, a member of lectin family, is glycosylated. A more likely explanation for their absence is low levels of these proteins in the MP preparation, present on MP but not detected by proteomics analysis.

Additionally, TF has been reported on MP by ELISA methods³⁰, but the majority of recent studies addressing MP Proteomics also had not detected TF.

Conclusions

In this study, complementary to our previous proteome analysis of MP in a large animal model of venous thrombosis, a diverse group of proteins associated with circulating MP in human patients presenting venous thrombosis were characterized. We can speculate that these proteins may influence thrombosis through inhibition of inflammation and MP generation (Gal3BP), coagulation cascade and fibrinolysis (A2M) and hemostatic plug formation (fibrinogen). New direct experimental studies evaluating the role of these proteins in thrombogenesis are warranted. Proteomic data from this investigation will be used to design upcoming studies aimed at identifying novel biomarkers and future target therapies for venous thrombosis.

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Table 1

Enriched and depleted proteins on DVT patients 2 out of 3 experiments.

Enriched proteins 2/3 experiments	IPI number*	i-TRAQ RATIOS
IgGFC-binding protein precursor	IPI00242956.4	2.61
Polymeric immunoglobulin receptor precursor	IPI00004573.2	1.77
CD5L antigen-like precursor	IPI00025204.1	1.76
Galectin-3-binding protein precursor (Gal3BP)	IPI00023673.1	1.76
Alpha-2 macroglobulin (A2M)	IPI00478003.1	1.57
13 kDa protein	IPI00657670.1	1.53
Immunoglobulin J chain	IPI00178926.2	1.27
Clusterin isoform 1 precursor	IPI00795633.1	1.25
C4A Complement component	IPI00643525.1	1.25
C4B Complement component	IPI00418163.3	1.25
C1QA Complement subcomponent subunit A precursor	IPI00022392.1	1.28
Depleted proteins 2/3 experiments	IPI number*	i-TRAQ RATIOS
Serpina1 Isoform 1 of Alpha-1-antitrypsin precursor	IPI00553177.1	0.79
Histidine-rich glycoprotein precursor	IPI00022371.1	0.74
Isoform 1 of Complement factor H precursor	IPI00029739.5	0.73
VWF 309 kDa protein	IPI00788786.1	0.70
Ig mu heavy chain disease protein	IPI00385264.1	0.70
Hemopexin precursor	IPI00022488.1	0.69
F2 Prothrombin precursor (Fragment)	IPI00019568.1	0.69
IGHG1 protein	IPI00815926.1	0.68
Serum amyloid P-component precursor	IPI00022391.1	0.68
doublecortin domain containing 5	IPI00418801.4	0.67
Fibrinogen beta chain precursor	IPI00298497.3	0.65
Isoform Gamma-B of Fibrinogen gamma chain precursor	IPI00021891.5	0.64
Transferrin variant, Serotransferrin precursor	IPI00798430.1	0.63
Isoform 2 of Fibrinogen alpha chain precursor; Isoform 1 of Fibrinogen alpha chain precursor	IPI00029717.1; IPI00021885.1	0.62
Extracellular matrix protein 1; Extracellular matrix protein 1 precursor	IPI00645849.1; IPI00003351.2	0.62
Afamin precursor	IPI00019943.1	0.61
Fibronectin 1 isoform 4 preproprotein	IPI00414283.5	0.59
IGHA1 protein	IPI00166866.3	0.59
Isoform 1 of Serum albumin precursor	IPI00745872.2	0.52
Isoform 3 of Fibronectin precursor; Isoform 1 of Fibronectin precursor	IPI00339223.1; IPI00022418.1	0.37
Rheumatoid factor RF-IP16 (Fragment)	IPI00827646.1	0.34
Lambda-chain precursor	IPI00827875.1	0.97

* IPI = international protein index

Table 2

Enriched and depleted proteins on DVT patients 3 out of 3 experiments and their function on coagulation and inflammation.

Enriched proteins 3/3 experiments	IPI number*	i-TRAQ RATIOS
Galectin-3-binding protein precursor (Gal3BP)	IPI00023673.1	1.76
Alpha-2 macroglobulin (A2M)	IPI00478003.1	1.50
Depleted proteins 3/3 experiments	IPI number*	i-TRAQ RATIOS
Serpina1 Isoform 1 of Alpha-1-antitrypsin precursor	IPI00553177.1	0.79
Histidine-rich glycoprotein precursor	IPI00022371.1	0.76
Hemopexin precursor	IPI00022488.1	0.69
Fibrinogen beta chain precursor	IPI00298497.3	0.65
Isoform Gamma-B of Fibrinogen gamma chain precursor	IPI00021891.5	0.64
Transferrin variant; Serotransferrin precursor	IPI00798430.1	0.63
Fibronectin 1 isoform 4 preproprotein	IPI00414283.5	0.59
Isoform 1 of Serum albumin precursor	IPI00745872.2	0.52
Rheumatoid factor RF-IP16 (Fragment)	IPI00827646.1	0.34

Table 3

All MP protein identified on the proteomics analysis

Protein	IPI
1. 13 kDa protein; APOC3 Apolipoprotein C-III precursor	IPI:IPI00657670.1; IPI:IPI00021857.1
2. Alpha-2-macroglobulin (A2M)	IPI:IPI00478003.1
3. Anti-(ED-B) scFV (Fragment); - Anti-(ED-B) scFV (Fragment)	IPI:IPI00869118.1; IPI:IPI00828062.1
4. Anti-folate binding protein (Fragment)	IPI:IPI00828190.1
5. FLJ27059 fis, clone SPL00869	IPI:IPI00442529.1
6. HRV Fab 026-VL (Fragment)	IPI:IPI00827510.1
7. Ig heavy chain V-II region SESS precursor	IPI:IPI00385557.1
8. Ig heavy chain V-III region GA	IPI:IPI00382483.1
9. Ig kappa chain V-I region AG	IPI:IPI00387022.1
10. Ig kappa chain V-I region Ni	IPI:IPI00387106.1
11. Ig kappa chain V-II region MIL	IPI:IPI00387110.1
12. Ig kappa chain V-III region B6	IPI:IPI00387113.1
13. Ig kappa chain V-III region NG9 precursor (Fragment)	IPI:IPI00387116.1
14. Ig kappa chain V-III region VH precursor	IPI:IPI00829834.1
15. Ig kappa chain V-IV region B17 precursor	IPI:IPI00386133.1
16. Ig mu heavy chain disease protein	IPI:IPI00385264.1
17. IgG VH protein precursor (Fragment)	IPI:IPI00383629.1
18. IGHV4-31 protein	IPI:IPI00784810.1
19. Immunoglobulin heavy chain variable region	IPI:IPI00477804.3
20. Immunoglobulin heavy variable 4-31	IPI:IPI00785084.1
21. Lambda-chain precursor	IPI:IPI00827875.1
22. Myosin-reactive immunoglobulin kappa chain variable region (Fragment)	IPI:IPI00384402.1
23. Rheumatoid factor light chain variable region precursor (Fragment)	IPI:IPI00827486.1
24. Rheumatoid factor RF-IP16 (Fragment)	IPI:IPI00827646.1
25. Transthyretin; TTR 13 kDa protein; TTR Transthyretin precursor	IPI:IPI00855916.1; IPI:IPI00646384.1; IPI:IPI00022432.1
26. V<gamma>1 protein (Fragment)	IPI:IPI00816409.1
27. Variable immunoglobulin anti-estradiol heavy chain (Fragment)	IPI:IPI00827581.1
28. Alpha 1B-glycoprotein; A1BG 41 kDa protein; A1BG Alpha-1B-glycoprotein precursor	IPI:IPI00745089.2; IPI:IPI00644018.1; IPI:IPI00022895.7
29. Afamin precursor	IPI:IPI00019943.1
30. Isoform 1 of Serum albumin precursor	IPI:IPI00745872.2
31. AMBP protein precursor	IPI:IPI00022426.1
32. Serum amyloid P-component precursor	IPI:IPI00022391.1
33. Apolipoprotein A-I precursor	IPI:IPI00021841.1
34. Apolipoprotein A-II precursor	IPI:IPI00021854.1
35. Apolipoprotein E precursor	IPI:IPI00021842.1
36. apolipoprotein L1 isoform a precursor	IPI:IPI00852826.1

Protein	IPI
37. Isoform 1 of Apolipoprotein-L1 precursor; APOL1 Isoform 2 of Apolipoprotein-L1 precursor	IPI:IPI00514475.4; IPI:IPI00186903.3
38. C10orf79 Isoform 1 of WD repeat-containing protein C10orf79; C10orf79 hypothetical protein LOC80217	IPI:IPI00478993.4; IPI:IPI00329556.6
39. C1QA Complement C1q subcomponent subunit A precursor	IPI:IPI00022392.1
40. C1QB Complement component 1, q subcomponent, B chain (Fragment); C1QB complement component 1, q subcomponent, B chain precursor	IPI:IPI00643948.2; IPI:IPI00477992.1
41. Complement C1q subcomponent subunit C precursor	IPI:IPI00022394.2
42. C1R;C17orf13;RP11-114H20.1;LOC442122;ACYPI Complement C1r subcomponent precursor	IPI:IPI00296165.5
43. Complement C1s subcomponent precursor	IPI:IPI00017696.1
44. Complement C2 precursor (Fragment)	IPI:IPI00303963.1
45. Complement C3 precursor (Fragment)	IPI:IPI00783987.2
46. Complement component 4A	IPI:IPI00643525.1
47. C4B C4B1	IPI:IPI00418163.3
48. C4BPA C4b-binding protein alpha chain precursor	IPI:IPI00021727.1
49. C4BPB Isoform 2 of C4b-binding protein beta chain precursor; C4BPB Isoform 1 of C4b-binding protein beta chain precursor	IPI:IPI00555752.2; IPI:IPI00025862.2
50. Complement C5 precursor	IPI:IPI00032291.1
51. Complement component C6 precursor	IPI:IPI00009920.2
52. Complement component C8 alpha chain precursor	IPI:IPI00011252.1
53. Complement component C8 beta chain precursor	IPI:IPI00294395.1
54. Complement component 8, gamma polypeptide; C8G Complement component C8 gamma chain precursor	IPI:IPI00513935.1; IPI:IPI00011261.2
55. Complement component C9 precursor	IPI:IPI00022395.1
56. CD5 antigen-like precursor	IPI:IPI00025204.1
57. B-factor, properdin; CFB Isoform 1 of Complement factor B precursor (Fragment)	IPI:IPI00639937.1; IPI:IPI00019591.1
58. Isoform 1 of Complement factor H precursor	IPI:IPI00029739.5
59. CLASP1 protein; Isoform 3 of CLIP-associating protein 1; Isoform 1 of CLIP-associating protein 1	IPI:IPI00828013.2; IPI:IPI00744551.2; IPI:IPI00396279.3
60. Clusterin isoform 1; CLU Clusterin precursor	IPI:IPI00795633.1; IPI:IPI00400826.1; IPI:IPI00291262.3
61. Ceruloplasmin precursor	IPI:IPI00017601.1
62. Doublecortin domain containing 5	IPI:IPI00418801.4
63. DENND1A 111 kDa protein; DENND1A Isoform 1 of DENN domain-containing protein 1A	IPI:IPI00873295.1; IPI:IPI00170641.3
64. Dystrophin Dp427p1 isoform; DMD Isoform 3 of Dystrophin; DMD Isoform 4 of Dystrophin	IPI:IPI00472316.1; IPI:IPI00304639.2; IPI:IPI00006091.2
65. DNAJC11 DnaJ (Hsp40) homolog, subfamily C, member 11	IPI:IPI00477887.1
66. Extracellular matrix protein 1; ECM1 Extracellular matrix protein 1 precursor	IPI:IPI00645849.1; IPI:IPI00003351.2
67. Translation initiation factor eIF-2B subunit beta	IPI:IPI00028083.1
68. Coagulation factor XII precursor	IPI:IPI00019581.1
69. A1 Coagulation factor XIII A chain precursor	IPI:IPI00297550.8
70. Coagulation factor XIII B chain precursor	IPI:IPI00007240.2

Protein	IPI
71. F2 Prothrombin precursor (Fragment)	IPI:IPI00019568.1
72. FAM135B hypothetical protein LOC51059	IPI:IPI00852662.1
73. FAM75A7;FAM75A1 Family with sequence similarity 75, member A1	IPI:IPI00845457.1
74. IgGFC-binding protein precursor	IPI:IPI00242956.4
75. Isoform 1 of Ficolin-3 precursor	IPI:IPI00293925.2
76. Isoform 2 of Ficolin-3 precursor	IPI:IPI00419744.4
77. Isoform 1 of Fibrinogen alpha chain precursor	IPI:IPI00021885.1
78. Isoform 2 of Fibrinogen alpha chain precursor; FGA Isoform 1 of Fibrinogen alpha chain precursor	IPI:IPI00029717.1; IPI:IPI00021885.1
79. Fibrinogen beta chain precursor	IPI:IPI00298497.3
80. Isoform Gamma-B of Fibrinogen gamma chain precursor	IPI:IPI00021891.5
81. fibronectin 1 isoform 4 preproprotein	IPI:IPI00414283.5
82. Isoform 1 of Fibronectin precursor	IPI:IPI00022418.1
83. Isoform 3 of Fibronectin precursor; FN1 Isoform 1 of Fibronectin precursor	IPI:IPI00339223.1; IPI:IPI00022418.1
84. Isoform 2 of GRIP and coiled-coil domain-containing protein 2	IPI:IPI00333197.4
85. Glutamate [NMDA] receptor subunit epsilon-1 precursor	IPI:IPI00029768.1
86. Alpha 2 globin variant (Fragment); HBA2;HBA1 Hemoglobin subunit alpha	IPI:IPI00853068.1; IPI:IPI00410714.5
87. Hemoglobin subunit beta	IPI:IPI00654755.3
88. Hemoglobin subunit delta	IPI:IPI00473011.3
89. Huntingtin	IPI:IPI00002335.1
90. Haptoglobin precursor	IPI:IPI00641737.1
91. Isoform 2 of Haptoglobin-related protein precursor; HPR Isoform 1 of Haptoglobin-related protein precursor	IPI:IPI00607707.1; IPI:IPI00477597.1
92. Hemopexin precursor	IPI:IPI00022488.1
93. Histidine-rich glycoprotein precursor	IPI:IPI00022371.1
94. Isoform 2 of Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial precursor; IDH3A Isoform 1 of Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial precursor	IPI:IPI00792971.2; IPI:IPI00030702.1
95. Insulin-like growth factor-binding protein complex acid labile chain precursor	IPI:IPI00020996.3
96. FLJ14473 fis, clone MAMMA1001080, highly similar to Homo sapiens SNC73 protein (SNC73) mRNA	IPI:IPI00386879.1
97. IGHA1 protein	IPI:IPI00166866.3
98. IGHA2 protein	IPI:IPI00783993.1
99. IGHD protein	IPI:IPI00418422.3
100. IGHG1 protein	IPI:IPI00815926.1
101. IGHG3 protein	IPI:IPI00472345.1
102. Ig gamma-4 chain C region; IGHG4 protein	IPI:IPI00829814.1; IPI:IPI00550640.2
103. IGHM protein	IPI:IPI00472610.2
104. immunoglobulin J chain	IPI:IPI00178926.2
105. IGKC protein	IPI:IPI00430847.1
106. Ig kappa chain V-II region GM607 precursor (Fragment)	IPI:IPI00743389.1
107. IGKV2-24 protein	IPI:IPI00440577.3

Protein	IPI
108. Similar to Ig kappa chain V-III region VG precursor	IPI:IPI00784430.3
109. Myosin-reactive immunoglobulin light chain variable region	IPI:IPI00549330.4
110. IGL@ protein	IPI:IPI00829640.1
111. IGLV2-14 protein	IPI:IPI00816555.1
112. IGLV3-21 protein	IPI:IPI00815938.1
113. V2-19 protein; IGLV3-16 V2-11 protein	IPI:IPI00552852.2; IPI:IPI00552771.2
114. Zinc finger protein Pegasus	IPI:IPI00154612.3
115. Inter-alpha-trypsin inhibitor heavy chain H1 precursor	IPI:IPI00292530.1
116. Inter-alpha (Globulin) inhibitor H2; ITIH2 Inter-alpha-trypsin inhibitor heavy chain H2 precursor	IPI:IPI00645038.1; IPI:IPI00305461.2
117. Isoform 1 of Kinesin-like protein KIF21B; KIF21B Isoform 2 of Kinesin-like protein KIF21B	IPI:IPI00872997.1; IPI:IPI00644778.2
118. Isoform HMW of Kininogen-1 precursor	IPI:IPI00032328.2
119. Keratin, type I cytoskeletal 9	IPI:IPI00019359.3
120. Laminin subunit gamma-1 precursor	IPI:IPI00298281.3
121. Galectin-3-binding protein	IPI:IPI00023673.1
122. PTD016 protein; LOC51136 CDNA FLJ25783 fis, clone TST06726	IPI:IPI00297702.4; IPI:IPI00007646.5
123. LOC646903 protein	IPI:IPI00061475.4
124. LOC729628 similar to Hornerin	IPI:IPI00787795.1
125. Isoform Long of Alpha-mannosidase IIx	IPI:IPI00027703.1
126. LOC730410;HLA-C;HLA-B MHC class I antigen	IPI:IPI00816057.1
127. Alpha-1-acid glycoprotein 1 precursor	IPI:IPI00022429.3
128. Alpha-1-acid glycoprotein 2 precursor	IPI:IPI00020091.1
129. Isoform 2 of Prolyl 4-hydroxylase subunit alpha-1 precursor; Isoform 1 of Prolyl 4-hydroxylase subunit alpha-1 precursor	IPI:IPI00218682.1; IPI:IPI00009923.1
130. pecanex-like 3; PCNXL3 similar to pecanex-like 3 isoform 5 similar to pecanex-like 3 isoform 2	IPI:IPI00869136.1; IPI:IPI00787937.1; IPI:IPI00738957.2
131. Isoform 2 of N-acetylmuramoyl-L-alanine amidase precursor; Isoform 1 of N-acetylmuramoyl-L-alanine amidase precursor	IPI:IPI00394992.1; IPI:IPI00163207.1
132. Polymeric immunoglobulin receptor precursor	IPI:IPI00004573.2
133. Plasminogen precursor	IPI:IPI00019580.1
134. PROS1 80 kDa protein	IPI:IPI00873445.1
135. Pregnancy zone protein precursor	IPI:IPI00025426.1
136. Reticulocalbin-1 precursor	IPI:IPI00015842.1
137. Serum amyloid A protein precursor	IPI:IPI00552578.2
138. SERPINA1 Isoform 1 of Alpha-1-antitrypsin precursor	IPI:IPI00553177.1
139. SERPINA3 Alpha-1-antichymotrypsin precursor	IPI:IPI00550991.3
140. SERPINA3 Isoform 1 of Alpha-1-antichymotrypsin precursor; SERPINA3 Alpha-1- antichymotrypsin precursor	IPI:IPI00847635.1; IPI:IPI00550991.3
141. SERPINC1 protein	IPI:IPI00844156.2
142. SERPIND1 Heparin cofactor 2 precursor	IPI:IPI00292950.4
143. Isoform 2 of Sodium- and chloride-dependent transporter XTRP3; Solute carrier family 6, member 20 isoform 1	IPI:IPI00216628.1; IPI:IPI00152803.4

Protein	IPI
144. TF Transferrin variant (Fragment); TF Serotransferrin precursor	IPI:IPI00798430.1; IPI:IPI00022463.1
145. TGFB1-induced anti-apoptotic factor 1	IPI:IPI00294690.2
146. Isoform 8 of Titin	IPI:IPI00759542.1
147. Vitronectin precursor	IPI:IPI00298971.1
148. VWF 309 kDa protein	IPI:IPI00788786.1
149. VWF von Willebrand factor precursor	IPI:IPI00023014.1
150. H14 nuclear protein UKp68 isoform 4	IPI:IPI00168449.1
151. ZNF148 78 kDa protein; SLC12A8 Isoform 2 of Solute carrier family 12 member 8; SLC12A8 Isoform 1 of Solute carrier family 12 member 8; ZNF148 82 kDa protein	IPI:IPI00872544.1; IPI:IPI00867607.1; IPI:IPI00855723.2; IPI:IPI00747011.1

Table 4

DVT and negative patients, D-dimer levels and MP counts.

ID numbers	Initials	D-dimer levels (ng/ml)	Total MP number	Observations
DVT patients				
5038-003	MB	0.82	9667.5	
5038-006	KZ	1.20	2977.5	
5038-007	MJ	5.05	15765	
5038-009	BS	6.00	8920	
5038-012	GT	6.90	2032.5	
5038-013	RW	0.97	16237.5	
5038-015	RT	3.17	36592.5	
5038-017	LH	1.42	182125	2.96 (DVT positive D-dimer average level)
5038-019	VH	1.10	105945	42,251 (MP average count on DVT positive patients)
DVT Negative patients				
2460-002	BP	0.41	40435	
2460-004	YSH	0.32	110240	
2460-005	EA	2.36	57980	
2460-006	AR	5.68	59620	
2460-007	DCP	0.42	31580	
2460-008	DB	0.26	25815	
2460-009	AD	7.44		
2460-200	TB	0.58	52155	2.07 (DVT negative D-dimer average level)
2460-201	MC	1.19	18250	49,509 (MP average count on DVT negative patients)