MARIANE CRISTINA FLORES NASCIMENTO

"IDENTIFICAÇÃO DE PROTEÍNAS DIFEENCIALMENTE EXPRESSAS EM PACIENTES COM TROMBOSE VENOSA PROFUNDA"

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Tese de Doutorado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção de título de Doutor em Fisiopatologia Médica, área de concentração em Biologia Estrutural, Celular, Molecular e do Desenvolvimento.

ORIENTADORA: *Profa. Dra. Joyce Maria Annichino-Bizzacchi*

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RESUMO

A trombose venosa profunda (TVP) é uma doença multifactorial, e possui uma alta taxa de morbi-mortalidade devido a complicações como embolia pulmonar e a síndrome pós-trombótica, e cerca de 25 % dos pacientes apresentarão recorrência em 5 anos. A identificação de novos fatores envolvidos na fisiopatologia da TVP pode ser convertida em implicações de grande importância no manejo destes pacientes, prevenção de recorrência e no desenvolvimento de novas terapias. O interesse em avaliar as plaquetas e as amostras de plasma se deve ao fato de que as funções plaquetárias não estão completamente compreendidas, e aparentemente o papel das plaquetas poderia ir além do seu envolvimento na hemostasia. Como o plasma potencialmente fornece uma janela para a observação do indivíduo como um todo, a análise proteômica tanto das plaquetas como do plasma poderia implementar o nosso conhecimento acerca da fisiopatologia da TVP. OBJETIVO: Neste estudo foram analisados os perfis proteicos de plaquetas e amostras de plasma de três pacientes com TVP, que foram comparados com os resultados obtidos a partir de amostras de 1 irmão e 1 vizinho para cada paciente, a fim de minimizar as interferências genéticas e ambientais. Estes pacientes apresentaram episódios espontâneos e recorrentes de TVP proximal e mencionaram um histórico familiar de distúrbios da coagulação. MÉTODOS: as plaquetas necessitaram ser lavadas e lisadas, e as amostras de plasmas tiveram a albumina depletada antes de as proteínas serem alquiladas, reduzidas, precipitadas com acetona e hidrolisadas com tripsina. Os peptídeos das plaquetas e das amostras de plasma foram fracionados por cromatografia de fase reversa e troca catiônica, respectivamente. Depois disto, os peptídeos plaquetários foram direcionados ao espectrômetro de massas LTQ-Orbitrap e a busca das proteínas foi realizadas através do Sorcerer/Sequest. Os peptídeos plasmáticos foram encaminhados ao espectrômetro de massas ESI Q-TOF Premier e as proteínas foram analisadas pelo Mascot. RESULTADOS: Cinco proteínas estiveram presentes apenas nas plaquetas dos pacientes, estando ausentes em todos os controles: a proteína ligante da Apolipoproteína A1, a sub-unidade ζ1 do Coatômero, a Desidrogenase 11-17-β do Estradiol, a Leucotrieno A-4 Hidrolase e a Sorbitol Desidrogenase. Além disso, verificou-se que outras proteínas estiveram diferencialmente expressas em amostras de plasma pacientes e controles: a protease C4-A, o inibidor C1 da Inter-α-Tripsina, o inibidor H1 de cadeia pesada, a proteína Amilóide Sérica A, a glicoproteína α-2-HS, a isoforma 2 da inter- α-tripsina, a apolipoproteína A-IV e o inibidor de cadeia pesada H4. CONCLUSÕES: A avaliação de plaquetas e amostras de plasma de pacientes com TVP espontânea permitiu a identificação de proteínas diferencialmente expressas quando comparados a irmãos e vizinhos, que podem desempenhar importantes papéis na fisiopatologia da doença por se relacionarem a processos inflamatórios, imunes e no de transporte de lipídeos.

Deep venous thrombosis (DVT) is multi-causal disease associated to a high morbi-mortality due to complications as pulmonary embolism and post-flebitic syndrome, and about 25 % of the patients present recurrence in 5 years. The identification of new factors involved to the physiopathology of DVT can be translated into important implications for the management of these patients, prevention of recurrence, and for the development of new therapies. We were interested about platelets and plasma because the platelets functions are not completely understood and apparently their role goes beyond a hemostatic player, and as the plasma potentially provides a window into the individual's state of health and disease, both could improve our knowledge about the DVT physiopathology. AIM: In this study we analyzed the protein profile of platelets and samples of plasma of 3 DVT patients and compared to results obtained from 1 sibling and 1 neighbor for each patient in order to minimize the genetic and environmental interferences. These patients presented spontaneous and recurrent episodes of proximal DVT and mentioned a familiar history of coagulation disorders. METHODS: the platelets needed to be washed and lysed, and the plasmas samples required albumin depletion before the proteins being alkylated, reduced, precipitated with acetone and hydrolyzed by trypsin. The peptides were fractionated by reverse phase and cation exchange liquid chromatography for platelets and plasmas samples, respectively. After that, the platelets peptides were directed to LTQ-Orbitrap mass spectrometer and the proteins search were performed by Sorcerer/Sequest. The plasma peptides went to ESI Q-TOF Premier mass spectrometer and the proteins were searched by

Mascot. RESULTS: We identified 5 proteins that were present on platelets from patients and absent in all the controls: Apolipoprotein A1 Binding-Protein, Coatomer (ζ1 sub-unit), Estradiol 11-17-β Dehydrogenase, Leukotriene A-4 Hydrolase and Sorbitol Dehydrogenase. In addition, we verified 6 proteins that were differently expressed between patients and controls: C4-A plasma protease, C1 inhibitor Inter-alpha-trypsin, inhibitor heavy chain H1, the serum amyloid A, alpha-2-HS-glycoprotein, isoform 2 of inter-alpha-trypsin, apolipoprotein A-IV and the inhibitor heavy chain H4. CONCLUSIONS: The evaluation of platelets and plasma samples from patients with spontaneous DVT allows the identification of proteins that are differently expressed when compared to siblings and neighbors, which can play important roles on the physiopathology of the disease due their relation to inflammatory, immune and lipid transportation.

3-Alpha-Diol: Androstan-3-Alpha,17-Beta-Diol

ACD: Acid-Citrate-Dextrosis Anticoagulant Solution

ACN: Acetonitrile

AlOX5AP: Arachidonate 5-Lipoxygenase-Activating Protein

aPL: Antiphospholipid Antibodies AT: Artherial Thrombosis

APOA1BP: Apolipoprotein A1 Binding Protein

AR: Aldose Reductase I/R: Ischemia-Reperfusion

BSA: Bovine Serum Albumin

COPI: Coat Protein Complex I

COPZ1: ζ1 Sub-Unit of Coatomer

CRP: C-Reactive Protein

DTT: Dithiothreitol

DVT: Deep Venous Thrombosis

ER: Endoplasmic Reticulum

ESI Q-TOF: LTQ: Eletrospray Quadrupole Time-of-Flight

HILIC: Hydrophilic Interaction Chromatography

HSD17B11: 11-17β Estradiol Dehydrogenase

IaIp: Inter-Alpha Inhibitor Proteins

IHRP: ITI Family Heavy Chain Related Protein

IL-6: Interleukin-6

ITI: Inter-Alpha-Trypsin Inhibitor

LA4H: Leukotriene A4 Hydrolase

LDL: Low Density Lipoprotein

LTB4: Leukotriene B4

LTQ: Linear Quadrupole Ion Trap

MAPK: Mitogen-Activated Protein Kinase

MI: Myocardial Infarction

MPS: Microparticles

NanoLC: Nano Liquid Chromatography

NO: Nitric Oxide

NOS: Nitric Oxide Synthase

PAPS: Primary Antiphospholipid Syndrome

PE: Pulmonary Embolism

PGE1: Prostacyclin

PPP: Platelet Poor Plasma

PRP: Platelet-Rich Plasma

PS: Protein S

RP-LC: Reverse Phase Liquid Chromatography

SAPS: Secondary Antiphospholipid Syndrome

SCX: Cation Exchange

SDS: Sodium Dodecyl Sulfate

SLE: Systemic Lupus Erythematosus

SORD: Sorbitol Dehydrogenase

TEV: Tromboembolismo Venoso

TFA: Trifluoroacetic Acid

TVP: Trombose Venosa Profunda

UPLC: Ultra Performance Liquid Chromatography

VTE: Venous Thromboembolism

SUMÁRIO

INTRODUÇÃO

1. Introdução

Trombose Venosa

A trombose é o processo patológico resultante da ativação e propagação inapropriada da resposta hemostática normal do organismo. Pode acometer qualquer sítio vascular, mas os membros inferiores são os mais comumente afetados. O tromboembolismo venoso (TEV) é considerado uma doença comum, com incidência anual de 1 a 3 casos por 1000 indivíduos (Silverstein *et al.,* 1998). Embora a incidência seja aparentemente similar entre homens e mulheres, há um incremento importante do risco com a idade, indo de 3 para 20 casos a cada 10.000 indivíduos entre pessoas de 30-49 e 70-79 anos, respectivamente (Fowkes *et al*., 2003).

A fisiopatologia da trombose baseia-se na Tríade de Virchow, composta por lesão vascular, alteração dos componentes sanguíneos e/ou estase venosa, sendo os dois últimos predominantes como fatores desencadeantes do TEV. Os efeitos agudos da trombose venosa profunda (TVP) podem ser locais, decorrentes da obstrução do retorno venoso, causando edema e dor no membro afetado. A embolia pulmonar é a sua complicação mais grave e está associada a uma alta morbi-mortalidade (Maffei *et al.,* 2001).

Até o momento, vários fatores de risco já foram relacionados à TVP. Os de natureza adquirida são representados por condições como imobilização, gravidez, câncer, anticoncepcional hormonal, trauma, cirurgia, tabagismo, síndrome do anticorpo antifosfolipídeo, entre outros (Rosendaal, 1999; Robetorye e Rodgers, 2001). Já os fatores hereditários decorrem de defeitos genéticos em um ou mais componentes da hemostasia, como na deficiência das proteínas C, S, antitrombina, na mutação G1691A no gene do fator

V (fator V de Leiden), na mutação G20210A no gene da protrombina e na disfibrinogenemia. Nos últimos anos, outras anormalidades da coagulação têm sido descritas como potencialmente trombogênicas, como níveis elevados de alguns fatores da coagulação, como o fibrinogênio e os fatores VIII, IX e XI (Rosendaal, 2005). No Brasil, alguns estudos demonstraram que a trombofilia hereditária também está presente em pacientes com TVP (Arruda *et al.,* 1995; Arruda *et al.,* 1997; Seixas *et al.,* 1998; Torresan *et al.,* 2000; Morelli *et al.,* 2002). Um estudo recente realizado em nosso laboratório mostrou que apenas o aumento do fator VIII é um fator de risco independente para a TVP na população brasileira (Mello *et al.,* 2009).

Uma nova teoria, no que se refere aos fatores de riscos para trombose venosa ou arterial, vem emergindo: a teoria "da última gota que faz transbordar o copo". Nela, se infere que indivíduos portadores de importantes fatores riscos (genéticos e/ou adquiridos) muitas vezes necessitariam de "gatilhos", ou fatores desencadeadores do evento trombótico. Estes fatores, muitas vezes compostos por atividades rotineiras, como ataques de tosse e espirros, migrânea, etc, seriam capazes de desencadear um episódio de TVP na presença de um ou mais fatores de riscos. A tabela 1, publicada por Lippi *et al* (2010), cita alguns exemplos de fatores de riscos do TEV, e os classifica como hereditários, adquiridos, predisponentes ou desencadeadores.

Tabela 1: Fatores hereditários, adquiridos, predisponentes, desencadeadores e sua relação com o tromboembolismo venoso

Fator	Herdado	Adquirido	Predisponente	Desencadeador
Deficiência de antitrombina	✓		✓	
Deficiência de Proteína C	✓		✓	

Deficiência de Proteína S	✓		✓	
Resistência à proteína C ativada	✓	✓	✓	
Mutação no gene da protrombina	✓		✓	
Níveis aumentados dos fatores da coagulação	✓	✓	✓	
Hiperhomocisteinemia	✓	✓	✓	
Obesidade	✓	✓	✓	
Imobilização prolongada		✓		✓
Viagens a longa distância		✓	✓	
Cirurgia		✓		✓
Gravidez		✓	✓	
Contraceptivos orais		✓	✓	
Terapia de reposição hormonal		✓	✓	
Falência cardíaca congestiva		✓	✓	
Trauma		✓	✓	
Anticoagulante lúpico		✓	✓	
Câncer		✓	✓	
Ataques de espirro e tosse		✓		✓
Migrânea		✓		✓
Relações sexuais		✓		✓
Exercícios físicos extenuantes		✓		✓
Abuso de drogas		✓		✓
Defecação		✓		✓

Por se tratar de uma doença multifatorial, a expressão clínica da TVP é muito variável e dependente de inter-relações genéticas e adquiridas (Liu *et al.,* 2005). O elo entre o genótipo e o fenótipo nem sempre é algo óbvio, sendo desafiador verificar como e quanto os genes relacionam-se entre si e com o meio ambiente, e o seu impacto sobre a fisiopatologia da doença (Mann *et al*., 2004; Lippi e Favarolo, 2009).

Mesmo com o conhecimento acerca da influência de todos estes fatores de riscos sobre o processo trombótico, entre 20 e 30 % dos pacientes com TVP não apresentam nenhum fator causador, sendo estes casos então classificados como TVP espontânea. Esta é uma situação intrigante, que leva a comunidade científica a inferir que ainda possam existir fatores de riscos a serem descobertos (Seligsohn e Lubetsky, 2001).

Além disso, a TVP é considerada uma doença crônica, e em 5 anos, aproximadamente 25 % dos pacientes apresentarão recorrência (Christiasen *et al.,* 2005; Prandoni *et al.,* 1996; Hanson *et al.,* 2000). Assim, a identificação dos pacientes com maior risco de uma nova trombose tem grande impacto e aplicabilidade na prática clínica.

Proteômica

A proteômica emergiu em meados de 1990, quando o termo foi introduzido por Wilkins e Williams, indicando a complementaridade da produção de 'PROTEínas' expressas pelo 'genOMA' de células, tecidos ou organismos inteiros (Wilkins, 1996). A descrição do genoma formou a base para sedimentar a compreensão dos mecanismos celulares e moleculares (Lander *et al.,* 2001; Venter *et al.,* 2001; Waterston *et al.,* 2002). Contudo, como não há necessariamente uma relação direta entre a sequência genômica, padrão de transcrição e expressão proteica (Gygi *et al.,* 1999), a análise do nível proteico propriamente dito tornou-se imprescindível na elucidação dos mecanismos biológicos.

Em um indivíduo, o genoma se mantém razoavelmente constante, mas as proteínas que ele codifica são modificadas durante e após sua tradução como resultado de interações bioquímicas com o meio ambiente. Apesar de sua complexidade, ferramentas de análise do proteoma já possibilitaram o descobrimento de muitas proteínas únicas e grupos de proteínas (bioassinaturas) que estão associadas tanto ao processo biológico normal como também às desordens patológicas (Speicher, 2000). Assim, estas proteínas podem servir como marcadores de doença. Alguns exemplos de sucesso no emprego da proteômica em doenças foram a identificação de marcadores de neoplasia de bexiga (Ostergaard *et al.,* 1999) e de mama (Page *et al.,* 1999). Além disso, as proteínas identificadas através da proteômica podem se tornar alvo para a produção de novos medicamentos. É importante ressaltar que mais de 80 % de todos os medicamentos disponíveis agem sobre proteínas (Drews, 2000).

Além da identificação de proteínas, esta ferramenta possibilita estimar a quantidade de uma ou mais proteínas, permitindo a identificação de proteínas sub e superexpressas. A análise das modificações pós-traducionais também pode ser realizada, e requer a análise de todos os peptídeos que apresentem um peso molecular não esperado. A fosforilação, a glicosilação, a sulfatação, dentre outras, são modificações extremamente importantes na determinação da atividade, estabilidade, localização e *turnover* de proteínas nos processos celulares. Além disso, o conteúdo proteico secretado de células em cultura nas mais diversas situações, ou "secretoma", também vem somando informações a este já tão vasto e desafiador campo de estudo.

Hemostasia, TVP e Proteoma

As plaquetas têm um importante papel na hemostasia, assim como nos processos de cicatrização e inflamação, tanto em condições normais, como em situações patológicas. A maioria dos estudos proteômicos de plaquetas podem ser agrupados em duas subcategorias: na catalogação global das proteínas presentes em plaquetas não estimuladas (McRedmond *et al*., 2004; O'Neill *et al.,* 2002) e em resposta à estímulos (Garcia *et al.,* 2004; Marcus e Meyers*,* 2004; Maguire *et al.,* 2002; Coppinger *et al.,* 2004; Gevaert *et al.,* 2000).

Garcia e colaboradores (2004) identificaram proteínas até então não descritas em plaquetas, bem como moléculas reacionais, como a SH3GL1, demonstrando a utilidade desta ferramenta na identificação de novas proteínas. Um estudo baseado em 2-DE/MALDI-MS, LC/MS, LC-MS/MS catalogou mais de 300 proteínas liberadas de plaquetas depois de ativadas pela trombina, incluindo o inibidor plasmático C1 (C1-INH) e a calumenina (Coppinger *et al*., 2004).

Um outro estudo realizado em citosol de plaquetas não estimuladas usando 2-DE-GE em uma faixa larga de pI (3–10) identificou 186 proteínas fosforiladas (Marcus *et al.,* 2004). Isto representou um aumento de dez vezes no número encontrado em estudos anteriores, que não haviam utilizado a espectrometria de massas.

Piersma e colaboradores (2009) analisaram peptídeos liberados após a ativação do receptor da trombina em indivíduos saudáveis usando LC-MS/MS, e identificaram 716 proteínas, incluindo proteínas liberadas dos grânulos e proteínas presentes em micropartículas geradas pela ativação.

Na última década, também foram conduzidos estudos para identificação do perfil protéico plaquetário em trombopatias hereditárias, como a Trombastenia de Glanzmann e a Síndrome das Plaquetas Cinzentas (Gnatenko *et al.,* 2006; Franchini *et al.,* 2009). Este tipo de análise promoveu a identificação de novos sítios de glicosilação neste tipo de patologia (Nurden e Nurden, 2002). Além disso, a identificação da expressão diferencial de proteínas plaquetárias tem o potencial de agregar informações sobre o processo de ativação e formação do trombo.

Srivastava e Dash (2001) realizaram medidas de anisotropia de fluorescência na membrana de plaquetas de pacientes com antecedente de AVC e observaram um aumento significativo da sua microviscosidade. Além disso, através do emprego de géis SDS e *immunoblotting*, puderam verificar que em plaquetas não ativadas desses pacientes, proteínas de 131, 100, 47 e 38 kDa mantiveram-se fosforiladas em sítios de tirosina.

Em relação ao estudo das trombofilias, Corral e colaboradores (2004) investigaram através da proteômica o efeito de mutações no gene da antitrombina sobre a proteína. Verificou-se que peptídeos trípticos de uma variante da antitrombina (P80S) conferiam uma estabilidade aumentada à proteína, ao ponto de ela não se polimerizar mesmo após incubação prolongada. Assim, esta variante não seria eficientemente secretada do hepatócito, e seus níveis estariam diminuídos no plasma.

Em busca de elucidar o mecanismo envolvido no aumento do risco de TEV em pacientes com a mutação G20210A no gene da protrombina, Gelfi e coraboradores (2004) empregaram a eletroforese de géis 2D e ESI, e observaram que esta simples troca de nucleotídeos está associada a uma maior glicosilação da protrombina, o que aumentaria a sua estabilidade.

Svensson e seu grupo (2006) analisaram se proteínas plasmáticas de baixo peso molecular (entre 500 e 20.000 Da) poderiam ser úteis no estudo de uma família com deficiência tipo 1 da proteína C, comparando o perfil dos indivíduos saudáveis e dos acometidos por episódios de TEV antes dos 40 anos. Observou-se uma diferença nos perfis, que permitiram a distinção do risco de TVP antes dos 40 anos com sensibilidade de 67 % e especificidade de 86 %. Os autores consideraram que a abordagem proteômica empregada seria útil na identificação de familiares mais predispostos a desenvolver complicações tromboembólicas.

Já Davis e Patterson (2007) determinaram o efeito da coagulação do sangue em tempos definidos (0, 15, 30, 45 e 60 minutos após a coleta) e verificaram uma significativa alteração nos peptídeos de baixo peso molecular (<10 kD). Um primeiro pico, presumido como sendo o fator 4 plaquetário, apareceria rapidamente após a ativação da hemostasia e diminuiria ao longo do tempo, seguido pelo aparecimento de quatro outros picos, todos fragmentos da cadeia α do fibrinogênio.

Estes resultados demonstram que a proteômica é uma área bastante atraente, com capacidade de agregar informações sobre o papel de diversos componentes da hemostasia, e sob várias condições.

Tendo em vista que os mecanismos celulares e moleculares que participam da fisiopatologia do TEV compõem uma área da pesquisa ainda com muitas questões em aberto, e que mesmo com a identificação de inúmeros fatores de risco, entre 20 e 30 % dos pacientes com TVP não apresentam nenhum fator determinado, estima-se que ainda existam fatores de risco a serem descobertos. Adicionalmente, mesmo pacientes com uma mesma alteração genética têm apresentação clínica muito diversa, sugerindo uma possível interação com outros fatores no desencadeamento da TVP. Além disso, a TVP é considerada uma doença crônica, e em 5 anos, aproximadamente 25 % dos pacientes apresentarão recorrência. Assim, a descoberta de novos biomarcadores, capazes de reconhecer os pacientes com maior risco de uma nova TVP, teria uma grande aplicabilidade na prática clínica.

OBJETIVOS

Os mecanismos celulares e moleculares que participam da fisiopatologia do TEV compõem uma área da pesquisa ainda com muitas questões em aberto. Mesmo com a identificação de inúmeros fatores de risco, entre 20 e 30% dos pacientes com TVP não apresentam nenhum fator determinado. Por isso, estima-se que ainda haja fatores de risco a serem descobertos. Adicionalmente, mesmo pacientes com uma mesma alteração genética podem ter uma apresentação clínica muito diversa, sugerindo uma possível interação com outros fatores no desencadeamento da TVP. Além disso, a TVP é considerada uma doença crônica, e em 5 anos, aproximadamente 25% dos pacientes apresentarão recorrência. Assim, medidas que identifiquem os pacientes com maior risco de uma nova TVP têm grande aplicabilidade na prática clínica.

Objetivos Gerais

Definir o perfil protéico de plaquetas e amostras de plasma de pacientes com TVP e compará-lo com o perfil do mesmo material proveniente de familiares e não familiares saudáveis.

Objetivos Específicos

- Estabelecer um protocolo eficiente de separação e lavagem de plaquetas, além da extração de suas proteínas totais;
- Estabelecer um protocolo eficiente de depleção de albuminas de amostras de plasmas;

- Otimizar a metodologia de troca catiônica e fase reversa para fracionamento dos peptídeos de plaquetários e plasmáticos;
- Identificar estas proteínas diferencialmente expressas empregando técnicas de espectrometria de massas em ambos os materiais.

RESULTADOS

RESULTADOS

CAPÍTULO 1:

"Metabolic And Inflammatory Proteins Differently Expressed In Platelets From Deep Venous Thrombosis Patients"

CAPÍTULO 2:

"Plasma Proteomics In Deep Venous Thrombosis Patients"

CAPÍTULO 1

“Metabolic And Inflammatory Proteins Differently Expressed In Platelets From Deep Venous Thrombosis Patients”

Artigo submetido:

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METABOLIC AND INFLAMMATORY PROTEINS DIFFERENTLY EXPRESSED IN PLATELETS FROM DEEP VENOUS THROMBOSIS PATIENTS

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Key words: Platelets, Deep Venous Thrombosis, Proteomics, Coatomer, Apolipoprotein A1 Binding Protein, Leukotriene A4 Hidrolase, 11-17β Estradiol Dehydrogenase, Sorbitol Dehydrogenase

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Abbreviations: VTE: Venous Thromboembolism; PE: Pulmonary Embolism; DVT: Deep Venous Thrombosis; MPS: Microparticles; ACD: Acid-Citrate-Dextrosis Anticoagulant Solution; PRP: Platelet-Rich Plasma; PGE1: Prostacyclin; SDS: Sodium Dodecyl Sulfate; DTT: Dithiothreitol; TFA: Trifluoroacetic Acid; ACN: Acetonitrile; HILIC: Hydrophilic Interaction Chromatography; UPLC: Ultra Performance Liquid Chromatography; BSA: Bovine Serum Albumin; NanoLC: Nano Liquid Chromatography; RP-LC: Reverse Phase Liquid Chromatography; LTQ: Linear Quadrupole Ion Trap; COPZ1: ζ1 Sub-Unit of Coatomer; APOA1BP: Apolipoprotein A1 Binding Protein; LA4H: Leukotriene A4 Hydrolase; HSD17B11: 11-17β Estradiol Dehydrogenase; SORD: Sorbitol Dehydrogenase; COPI: Coat Protein Complex I, ER: Endoplasmic Reticulum; LDL: Low Density Lipoprotein; LTB4: Leukotriene B4; AlOX5AP: Arachidonate 5-Lipoxygenase-Activating Protein; PS: Protein S; NO: Nitric Oxide; NOS: Nitric Oxide Synthase; MAPK: Mitogen-Activated Protein Kinase; 3-Alpha-Diol: Androstan-3-Alpha,17-Beta-Diol; AR: Aldose Reductase; I/R: Ischemia-Reperfusion

SUMMARY

Deep venous thrombosis (DVT) is multi-causal disease associated to a high morbidity and mortality due to complications arising from increased risk for pulmonary embolism. . The identification of new factors of relevance to the physiopathology of DVT enhance our most basic understanding of disease mechanisms and can be translated into improved management of patients, prevention of recurrence and development of new therapies. The platelets functions are not completely understood, and apparently their role goes beyond a hemostatic player. AIM: In this study we analyzed the whole platelet protein profile of samples of 3 DVT patients and compared to results obtained from 1 sibling and 1 neighbor for each patient in order to minimize the genetic and environmental interferences. These patients presented spontaneous and recurrent episodes of proximal DVT and mentioned a familiar history of coagulation disorders. METHODS: the platelets were washed, lysed, and the proteins were alkylated, reduced, precipitated with acetone and hydrolyzed by trypsin. Peptides were first separated by HPLC and peptide fractions were further detected by LC-MS/MS. RESULTS: We identified 5 proteins that were present on patients and absent in all the controls: Apolipoprotein A1 Binding-Protein, Coatomer (ζ1 sub-unit), Estradiol 11-17-β Dehydrogenase, Leukotriene A-4 Hydrolase and Sorbitol Dehydrogenase. CONCLUSIONS: The analysis identified proteins that currently are not related to the physiopathology of DVT, which maybe of pathological importance for metabolic and inflammatory processes in DVT.

INTRODUCTION

The thrombosis is a multicausal pathology resulted from the activation and propagation of the hemostatic response, and can occur in any vascular site. Venous thromboembolism (VTE) is considered a common disease, and its incidence is from 1 to 3 cases per 1000 individuals (Silverstein *et al.,* 1998; Hanson *et al.,* 1997). A total of 30 % of patients suffering from VTE die within 30 days, a fifth from sudden death due to pulmonary embolism (PE), and 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 spontaneous deep venous thrombosis (DVT) (Heit, 2002). Therefore, the search for mechanisms involved to the physiopathology of DVT can be translated into important implications for the management of these patients, prevention of recurrence, and for the development of new therapies.

Traditional coagulation tests, especially clot-based assays, are useful for describing major abnormalities in the haemostatic response, but fail in their application to assessing 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. Thus, proteomic analysis may hold promise for characterizing or understanding biological pathways and pathophysiological interactions (Lippi *et al.,* 2010).

Until the moment, some abnormal proteins expression was identified by proteome approaches in patients with increased risk to develop DVT. New findings have been done, as the identification of one antithrombin variant (P80S) (Corral *et al*., 2004), which cannot be efficiently secreted from the hepatocyte, associated to loss of function. Gelfi and coworkers (2004) analyzed samples of pooled plasmas from carriers of the G20210A prothrombin mutation and observed an increased level of glycosylation on this protein, which confer a greater stability. Another study performed on low molecular weight (500–20,000 Da) proteins noticed that they could be useful differentiating members of a type I protein C deficiency family who have suffered thrombotic events before the age of 40 years from disease-free family members (Svensson *et al*., 2006).

The 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 (Munnix *et al.,* 2009). Studies in platelet cytosolic proteome made possible the identification of many platelet glycoproteins and proteic defects in patients with hereditary hemorrhagic diseases (Nurden e Nurden, 2002) and after stimuli response (Garcia *et al.,* 2004; Marcus and Meyer, 2004; Maguire *et al.,* 2002; Coppinger *et al.,* 2004; Gevaert *et al.,* 2000). The microparticles (MPs) from DVT patients were also studied, and increased expressed (galectin-3 binding protein and alpha-2 macroglobulin) and depleted (fibrinogen beta and gamma chain precursors) proteins could be identified (Ramacciotti *et al.,* 2010).

Alteration on the platelets protein profile has a potential to contribute to the pathogenesis of DVT. Here, we present a comprehensive proteomic profile of human platelets from patients with spontaneous and recurrent DVT, with the comparison to healthy siblings and neighbors. Our hypothesis was that the patient's platelets could present a different protein expression.

PATIENTS AND METHODS

Patients

Patients were recruited from the Outpatient Clinic of the Hematology and Hemotherapy Center of Campinas – UNICAMP. Samples were collected between February 2009 and September 2009. We selected patients 6 months after withdrawal of warfarin therapy, which diagnosis of DVT was confirmed by Doppler ultrasonography. 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 the proteic expression, two control groups composed by siblings and individuals originated from the same geographic area were included. The 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 antiplatelet drugs 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, Kremsmunster, Austria) containing EDTA and was directed to a blood count in order to avoid problems during analysis due its contamination with traces of collagen and thrombin. The blood directed to the platelets separation was collected with a wide–bore (16-gauge) needle with syringe containing 1 volume of acid-citrate-dextrosis anticoagulant solution (ACD; 85 mM citric acid, 66.6 mM trisodium citrate and 111 mM dextrose, pH 4.5), and were added with 6 volumes of blood as previously described by Cazenave and coworkers (2004). The platelets were isolated immediately after blood donation to avoid changes in their physiology and viability. After the blood samples were kept resting at 37 ºC for 15 min, they were centrifuged at 200 x g / 37 °C for 15 min to obtain the platelet-rich plasma (PRP). The lower third of the platelet rich plasma was left in the tube in order to avoid contamination with other blood cells such as erythrocytes and leukocytes. The PRP were separated and incubated at 37 °C for 15 min and then centrifuged at 2200 x g/37 °C for 12 min. The supernatants were discarded and the platelet pellets were ressuspended in Tyrode's albumin solution (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, pH 7.4) supplemented with 10 U/mL of heparin and 0.5 μM prostacylin (PGE1) (Sigma-Aldrich, St. Louis, MO, EUA). After 15 min incubation at 37 °C, 0.5 μM of PGE1 were added to the first wash and then centrifuged at 1900 x g for 8 min. This washing procedure was repeated and after that the platelets, erythrocytes and leucocytes were counted in an automatic analyzer. The platelet suspension was incubated again at 37 °C for 15 min, added of PGE1 0.5μM t and then centrifuged at 1900 g for 8 min. The platelet pellets were frozen in liquid nitrogen and stored at -80 °C.

Protein Extraction

The protein extraction and digestion were performed according to Wojcechowskyj and coworkers (2011). The platelets were taken out of -80 °C and put on ice. One mL of lysis buffer (SDS 0.3 %, 20 mM DTT in a tris buffer) plus 10 μL of protease inhibitor (P8465, Sigma-Aldrich, St. Louis, MO, EUA) was added to 50 μL of cell pellets and the samples were boiled in water for 5 min. Benzonase 10 % (Novagen®, Madison, WI – EUA) was added and the samples were let sit at room temperature for 5 min. Iodoacetamide (final concentration 50 mM) was added and let sit at room temperature on the dark for 30 min. After that, 5mL of acetone were added, the samples were vortexed for 5 min and left at -20 ºC for 2 hours. The aliquots were taken out of the -20 ºC and spin at 1500 x g at 5 ºC for 5 min. The supernatant was discarded and the process was performed twice using 5 mL of acetone 80 %. The proteins were immediately directed to the digestion process.

Protein *in-solution* digestion

The pellets were ressuspended in 200 μL of trypsin mix (10ng/μL Porcine Trypsin - Promega, Madison, WI, USA; 0.1 % Rapigest - Waters®, Milford, MA, USA in 30mM NH_4HCO_3) and were let rotate at room temperature for 1 hour. After that, they were put at 37 °C for 4 hours. The digestion was stopped with formic acid (2.5 % final concentration) and the samples were let sit at 37 ºC for 1 hour and spin at 20,000 x g for 20 min to pellet the rapigest. The samples were passed thru Sep-Pak columns (C18, Waters®, Milford, MA, USA) in order to remove salts. The process was performed according to the manufacturer. The columns were activated with 2 mL of acetonitrile (ACN) 100% and were equilibrated with 2 mL of 0.1 % trifluoroacetic acid (TFA). The samples were added slowly, the columns were washed with 2 mL of 0.1 % TFA and then eluted with 2 mL of 75 % ACN/0.1 % TFA. The samples were frozen in liquid nitrogen and lyophilized.

Hydrophilic interaction chromatography (HILIC)

The HILIC was performed according to McNulty e Annan (2008). The peptides were fractionated according to their hydrophilicity applying the column TSKgel Amide-80 21487, 1 mm x 25 cm, 5 Å (TOSOH Bioscience®, Tokyo, Japan) connected to the UPLC (Shimadzu Scientific®, Duisburg, German). The column was conditioned by multiple washes and runnings of 1 µg/µL of the standard (MassPREP BSA Digestion Standard, Waters®, USA) in ACN 90 %/0.1 % TFA.

As the peptides of the standard and from all the samples were lyophilized, they were ressuspended with 100 µL of 50 % ACN and centrifuged 30,000 rpm for 1 min. The supernatant were dosed using the Nanodrop (Thermo Scientific®, Waltham, MA, EUA), and 100 µg of peptides were re-lyophilized and ressuspended with 50 µL of 90 % ACN/ 0.1 % TFA. The peptides were fractionated according to their affinity with two different buffers: one hydrophilic (A: 0.1 % TFA) and one hydrophobic (B: 98 % ACN/0.1 % TFA). The analysis was performed with flow of 0.5 mL/min and initiated with 85 % of buffer B for the first 10 min, and its concentration had a gradual decrease over 65 min, with a concomitant increase of the 0.1 % TFA. From there, the buffer B concentration decreased from 70 to 10 % in 10 min and was kept in that condition for 10 minutes more, which finally returned to 85 % in the last 2 minutes (t=85 min). This made possible the gradual release of the peptides from the column, which were collected in 8 intervals of 10 minutes each, from t=5 to t=85 min.

Mass spectrometric analysis

After fractionation, the tryptic peptides were frozen in liquid nitrogen and then lyophilized and ressuspended in 40 µL of 0.1 % formic acid and directed to the hybrid mass spectrometer LTQ-Orbitrap (Thermofisher 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 (I.D.) × 20 cm ProteoPep (New Objective®, Woburn, MA, USA). The mobile phase A consisted of 1 % methanol/0.1 % formic acid and mobile phase B of 1 % methanol/0.1 % formic acid/79 % ACN. Peptides were eluted into the mass spectrometer at 300 nL/min with each RP- LC run comprising 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 1^6, while MSn AGC was 5^6, respectively. FTMS full scan maximum fill time was 500 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 raw data files were submitted to Sorcerer-SEQUEST (ver. 4.0.3, rev 11; SagenResearch, San Jose, CA). 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 output of the search was loaded into Scaffold 3 (Scaffold 3_00_3, Proteome Software Inc. Portland, OR, USA), and initially filtered using 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. SEQUEST sequence-to-spectrum assignments were analyzed by Scaffold (Proteome Software, Portland, OR, USA), the TransProteomic Pipeline (TPP ver. 4.0.2).

Semi quantitative analysis

The spectral counting is correlated with peptide abundance (Vogel and Marcotte, 2008) by counting the number of detected tryptic peptides and their corresponding MS spectra. However, spectral counting is confounded by the fact that peptide physicochemical properties severely affect MS detection resulting in each peptide having a different detection probability. Lu et al. (2007) described a modified spectral counting technique, Absolute Protein Expression (APEX), which improves on basic spectral counting methods by including a correction factor for each protein (called Oi value) that accounts for variable peptide detection by MS techniques. The technique uses machine learning classification to derive peptide detection probabilities that are used to predict the number of tryptic peptides expected to be detected for one molecule of a particular protein (Oi). This predicted spectral count is compared to the protein's observed MS total spectral count during APEX computation of protein abundances. Due this properties, the semi quantitative analysis was performed applying the Apex Quantitative Proteomics Tool (Version 1.1.0, Pathogen Functional Genomics Resource Center, Rockville, MD, USA) according to Lundgren *et al* (2010) and required the construction of one *arff*, one *oi* and finally one *apex* file, which estimates the amounts of each identified protein.

RESULTS

According to the criteria, from the 30 pre-selected subjects, 3 with clinically documented DVT were selected because they showed spontaneous and recurrent DVT, positive familiar history and appropriate controls were available. 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 amlodipine besylate and atenolol. All patients presented proximal DVT and the time between the last DVT event and the sample collection varied from 1 to 4 years. All the siblings and neighbors were matched to the patients as described on the methods section.

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

	N of males/females	**Mean age; min-max (years)**	**P**
Patients	1 / 2	46.33 ; 44-50	-
Siblings	1 / 2	49.67 ; 41-56	0.7
Neighbors	1 / 2	46.00 ; 40-50	0.83

In the blood count, the platelets count varied from 263 to 312, 185 to 365 and from 230 to 466 x 1000/μL between patients, siblings and neighbors respectively. Using previously established protocols the platelets from all individuals were separated, washed and pelleted in order to minimize the contamination with other cells or plasma. In the last step, the platelets were counted by a cell counter (Cell Dyn 1700, Abott® Laboratories, North Chicago, IL, USA), and the presence of contaminant leukocytes and erythrocytes was estimated to be ≤0.2 % of platelet population [leucocytes 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, further verifying that the contamination with these cells in the isolated platelets was minimal.

Applying the HILIC technology, the peptides obtained by the enzymatic digestion of the platelets proteins were successfully fractionated, enhancing the identification of the proteins after the mass spectrometry analysis. The figure 1 illustrates the chromatograms obtained on the peptides fractionation, resulted from the platelet sample of a DVT patient (figure A), his sibling (figure B) and his neighbor (figure C). In those figures, it is possible to observe the gradual decrease of the 98 % ACN/0.1 % TFA from the beginning until the middle of the process, as well as its sudden decrease and increase concentration in the end of the analysis, which allowed the gradual release of the peptides from the column. The figure D shows the superposition of the chromatograms, illustrating the interindividual differences that could be a consequence of different protein expression patterns between the analyzed group.

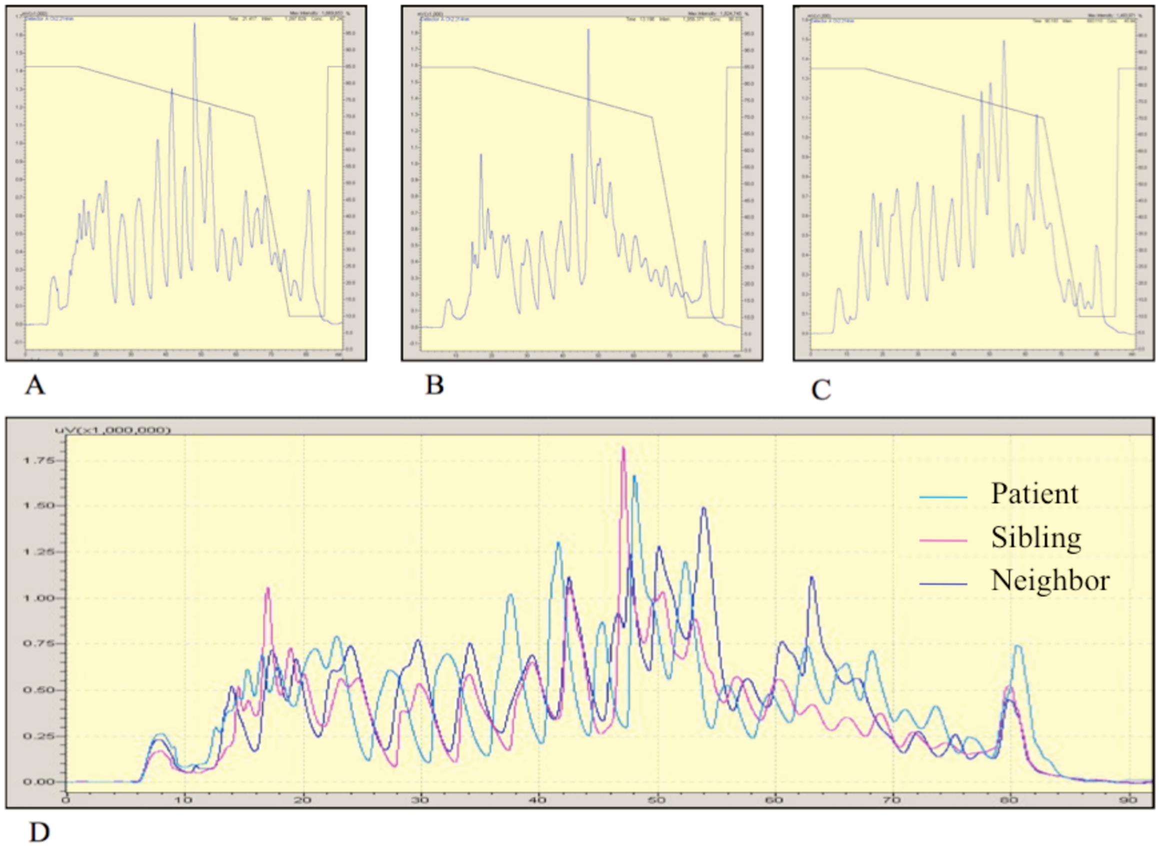

Figure 1: Chromatograms obtained on the peptides fractionation by reverse phase applying the UPLC in platelets from a DVT patient (figure A), his sibling (figure B) and his neighbor (figure C). The figure D shows the superposition of the chromatograms, illustrating the interindividual differences.

Mass spectrometry

The first analysis performed was the methodological variability applied on the proteins identification of all the subjects of 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 employed.

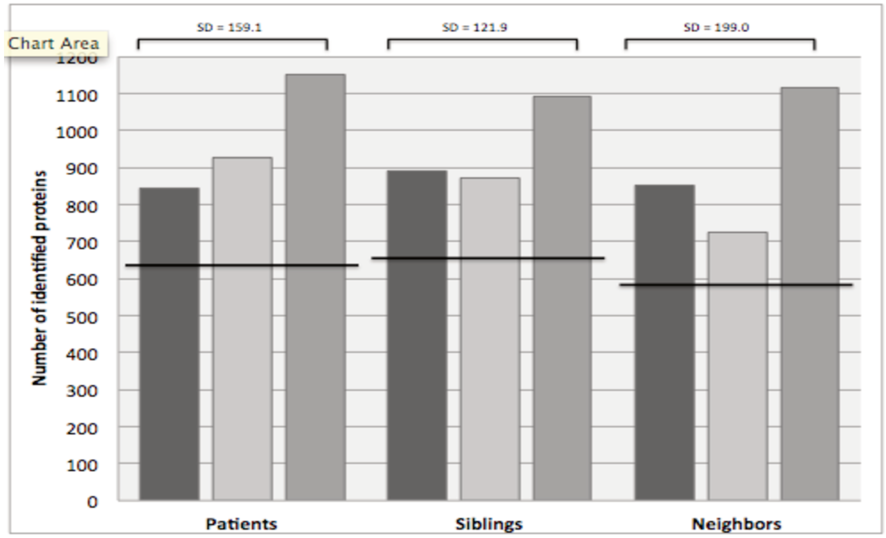

Figure 2: Number of proteins identified after mass spectrometry analysis of platelets tryptic peptides from 3 spontaneous DVT patients, their 3 siblings and 3 matched neighbors. The black line shows the number of common proteins identified between the analyzed groups.

The presence and absence of the proteins was evaluated by two different ways: proteins present on 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: ζ1 sub-unit of coatomer (COPZ1), apolipoprotein a1 binding protein (APOA1BP), leukotriene A4 hydrolase (LA4H); 11-17β estradiol dehydrogenase (HSD17β11) 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.

The analysis of the increased and decreased proteins was performed by Apex Software, and the data evaluation were performed as in the presence/absence analysis: after the separation of all the differentially expressed proteins ($p \geq 0.01$) in each individual, the proteins were compared between each patient with his sibling and neighbor, resulting in a list of different expressed protein per set of analysis. In the end, the results of each set were compared to the other two, and unfortunately no protein could be identified as a constant.

DISCUSSION

Platelets are the main mediators of haemostasis and thrombin generation, and there is no doubt about their relevance in the pathophysiology of VTE. Since platelets are readily available, are easily isolated in relatively large numbers, lack nuclei and genomic DNA, and have a limited RNA pool, proteomic techniques are ideally suited for the analysis of platelets. Toward this goal, we employed a proteomic approach in order to identify the platelets proteins from DVT patients and compare to profiles obtained from their siblings and neighbors.

The inclusions of two different kinds of controls in order minimize the genetic and environmental influence over the proteins expression increased the reliability and the level of exigency over the results as well. We attribute this fact over the lack of results on the differential expressed analysis by Apex. There were no proteins that were constantly increased or decreased between each patient with his controls and, after that, among all the patients.

The platelet proteome is subject to rapid changes in response to external signals, giving rise to potentially large within- and between subject variation (Gnatenko *et al.,* 2006). In this study, the methodology applied was carefully developed by Cazenave and coworkers (2004) in order to isolate the platelets with minimum activation and contamination with other cells and plasma. In this methodology, the blood was collected by syringe, minimizing the impact of the platelets to the tube, and the first tube was discarded due the high levels of thrombin generated by the lesion of the vessel wall. The anticoagulant ACD was chosen due its capacity of better prevents the platelet spontaneous activation (Pignatelli *et al.,* 1995). The wash buffer had glucose, physiologic pH and the prostacyclin (PGI3) was added in order to inhibit the platelet function by the decrease of expression of platelet fibrinogen receptors and P-selectin (Kozek-Langenecker *et al.,* 2003). The PGI3 is produced by the endothelial cells and actives the platelet protein G, which acts over the adenilciclase of the membrane. The adenilciclase converts ATP in cAMP, and the last one blocks the calcium release from the dense tubular system and the release of the triphosphate inositol and several kinases, preventing the platelet aggregation (Schrör K., 1997). The washes were intercalated with incubations at 37 ºC, which could revert initial activation processes.

During the analysis, it was identified 5 proteins that were only present on patients: the apolipoprotein A1 binding-protein, the ζ1 sub-unit of coatomer), the estradiol 11-17-β dehydrogenase, the leukotriene A-4 hydrolase and the sorbitol dehydrogenase. Those proteins were below the threshold of identification in controls: Part of them had relation with lipid transportation, metabolism and inflammation.

The platelets, for long time considered merely effectors of thrombosis, have been implicated in inflammatory conditions and on the pathobiology of these disease processes. Not only do 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 (Semple *et al.,* 2011). Activated platelets are involved in processes of leukocyte extravasation in response to inflammatory stimuli, increasing leukocyte rolling on a pro-inflammatory surface, utilizing different types of adhesion molecules, in a reciprocal cross-talk (Krishnan *et al.,* 2011). Sehgal and Storrie (2007) have identified two classes of α-granule: one that contains fibrinogen and another that contains von Willebrand factor. It is tempting to speculate that platelets may also differentially package pro-inflammatory and anti-inflammatory molecules into distinct granule subpopulations. If so, it would indicate that platelets may store immunomodulatory substances in a specific manner in order to respond to different types of tissue damage.

In the literature, the leukotriene family is mentioned as important players in immediate hyposensitivity reactions and inflammation. The **leukotriene A4 hydrolase (LA4H)** is a proinflammatory enzyme that generates the inflammatory mediator leukotriene B4 (LTB4). Qiu and collegues (2006) reported increased mRNA and protein levels of LA4H in 72 human carotid atherosclerotic plaques compared to 6 controls. The proteins co-localized within macrophages in intimal lesions, presumably facilitating enzyme coupling and leukotriene B4 (LTB4) synthesis. There was a correlation between increased levels of LA4H mRNA and recent or ongoing symptoms of plaque instability. Unstable vascular plaques showed abundant accumulation of activated neutrophils that produce leukotriene B4, which induces the myeloperoxidase. This protein generates potent oxidants such as hydrochloric acid, oxidizes LDL cholesterol and renders them proinflammatory (Naruko *et al.,* 2002, Zhang *et al.,* 2001, Schmitt *et al.,* 1999), inactivates protease inhibitors and consumes nitric oxide, all of which escalate the inflammatory response (Eiserich *et al.,* 2002). It is known that the vascular endothelial cells release nitric oxide, PGI2 and CD 39 as protector agents, inhibiting the platelet adhesion to the wall. Myeloperoxidase levels have been shown to be elevated in patients with diagnosed coronary artery disease and within atherosclerotic lesions that are prone to rupture (Zhang *et al.,* 2001; Sugiyama *et al.,* 2001). These findings were corroborated by Spanbroek and colleagues (2003), who investigated the 5-lipoxygenase cascade, which leads the formation of leukotrienes and exhibit strong proinflammatory activities in human atherosclerosis, and verified that the 5-lipoxygenase pathway was abundantly expressed in arterial walls of patients afflicted with various lesion stages of atherosclerosis of the aorta and of coronary and carotid arteries. The number of 5-lipoxygenase expressing cells markedly increased in advanced lesions.

Variants of the gene that encodes the arachidonate 5-lipoxygenase-activating protein (ALOX5AP) have been associated with risk of myocardial infarction (Helgadottir *et al*., 2004). In another study, the same group (2006) showed that a haplotype spanning the LA4H gene, which has the same biochemical pathway than ALOX5AP, confers risk of myocardial infarction in an Icelandic cohort. Neutrophils from myocardial infarction cases with the at-risk haplotype resulted in more LTB4 than controls. Hakonarson and coworkers (2005) performed a randomized, prospective, placebo-controlled, crossover trial of an inhibitor of 5-lipoxygenase–activating protein in myocardial infarction patients who carried the risk variants of the 5-lipoxigenase-activating protein gene or in the leukotriene A4 hydrolase gene and observed that the haplotype in the leukotriene B4 receptor complex was found to confer a 2.3-fold increase in risk of ischemic and cardioembolic stroke in England and German cohorts.

All those studies are in a completely accordance with the ours, and the presence of LA4H can indicate a sustained inflammatory status, 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 **11-17-β-estradiol dehydrogenase (11-17βED)** is an interesting protein that converts androstan-3-alpha,17-beta-diol (3-alpha-diol) to androsterone *in vitro* (Brereton *et al*., 2001), suggesting that it may participate in androgen metabolism during steroidogenesis. It may act by metabolizing compounds that stimulate steroid synthesis and/or by generating metabolites that inhibit it. This protein is localized on the ER membrane under normal conditions and is transferred to lipid droplets after the induction of its formation (Horiguchi *et al.,* 2008).

The involvement of this protein on the coagulation disorders is presently contradictory. While some data describs a protective effect, other studies imply deleterious effects.

Estrogen is known to have multiple protective effects on the cardiovascular system (Mendelsohn and Karas, 1999). Wang and colleagues (2002) also showed that 11-17βED deficiency leads to a decrease in total cardiac NOS activity due to changes in NOS3 activity. Cardiac myocytes and cardiac fibroblasts contain estrogen receptor isoforms, and they can regulate the cardiac expression of endothelial and inducible NO synthase and connexin 43 (Massion *et al.,* 2003). Estrogen also modulates the activity of the mitogen-activated protein kinase (MAPK) pathways in cardiac myocytes (Damy *et al.,* 2004). In fact, myocyte cross-sectional area was attenuated by estrogen treatment compared to placebo. Another study performed by van Eickels and coworkers (2001) supports the hypothesis that estrogens have direct effects on cardiac myocytes by the inhibition of the p38-MAPK phosphorylation and increase the expression of ventricular atrial natriuretic peptide, resulting in an antihypertrophic effect in our model of pressure overload.

However, the anticoncepcional use and the post-menopausal hormone therapy are established risk factors to DVT (Robetorye e Rodgers, 2001). Suzuki and coworkers (2010) investigated the molecular mechanisms behind the reduction in PS levels in pregnant woman. They observed that 11-17βE might modulates the ligand-dependent repression of the PS alpha gene and also the production of mRNA and antigen of PS in HepG2 and ER α cells. Cohen (2005) postulates that many effects of the postmenopausal hormone replacement therapy could be explained by the down regulation of 11-17βED, which reduces the tissue specific cortisol production and diminishes the endogenous anti-inflammatory effects, allowing the progression of both vascular and pulmonary inflammation.

The ζ1 sub-unit of the coatomer is a 700 kDa cytosolic protein complex consisting of seven equimolar subunits (α-, β-, β'-, γ-, δ-, ε- and ζ). The coatomer complex and the ADP-ribosylation factor 1 are the main components of coat protein complex I (COPI), which is involved in vesicle transport between the endoplasmic reticulum and the golgi apparatus (Daro *et al.,* 1997; Gu *et al.,* 1997; Whitney *et al.,* 1995), and for the retrograde Golgi-to-ER transport of dilysine-tagged proteins (Titorenko e Rachubinski, 2001). The perturbation in COPI increased the storage of triglycerides by decreasing the lipolysis rate (Beller *et al.,* 2008; Guo *et al.,* 2008). Furthermore, parallel studies in yeast and Drosophila S2 cells (Guo *et al.,* 2008) also suggested a role of coatomer function in lipid droplet regulation. Another study (Dekroon e Armati, 2001) verified that apolipoprotein E synthesis depends on the COPZ1 complex.

The complex also influences the Golgi structural integrity, protecting its membrane from uncontrolled fusion (Elazar *et al.,* 1994; Lippincott-Schwartz *et al.,* 1989; Scheel *et al.,* 1997; Guo *et al.,* 1994) by the endocytic recycling of LDL receptors (Passreiter *et al.,* 1998). Beller and coworkers (2008) found a positive regulation of lipolysis by the coatomer retrograde-vesicle trafficking pathway. He studied this protein by knockdowns of the complex members in Drosophila S2 and Kc167 cells, and in mouse 3T3-L1 and AML12 cells. Surprisingly, only the ε-subunit repeatedly failed to produce a lipid storage phenotype following RNAi in both the S2 (Guo *et al.,* 2008) and on the Kc167 cells.

The lipid metabolism and transportation maybe could present relevance on DVT. This is also corroborated by another protein associated with this function: the **apolipoprotein A-I Binding Protein (APOA1BP).** Ritter and coworkers (2002) confirmed direct binding between the apolipoprotein A1 (APOA1) and its binding protein (APOA1BP) by *in vitro* protein binding assays and co-purification of the 2 proteins from hepatoma cell lysates. The APOA is synthesized by human intestine and liver and its biological function is not completely understood. This protein is considered an anti-atherogenic protein due its participation as a constitutive part of the chylomicrons and HDL. The APOA1 would interact with the precursor beta HDL, accepting the free cholesterol released from tissues to the liver. APOA1 also would activate the lecithin cholesterol acil transferase (LCAL) as its cofactor, which is related with the esterification of the free cholesterol in HDL particles. In Budd-Chiari syndrome (BCS), a rare vascular liver disorder caused by thrombosis of the hepatic veins, the patients showed a significant decrease of APOA1 plasma levels (Talens *et al.,* 2011). In another interesting study, Nordøy and coworkers (2003) discovered that the fasting levels of activated FVII (FVIIa) and FVII-Ag correlated to serum triglycerides and APOA1. FVIIa and FVII:C increased during postprandial hyperlipemia, but the concentrations of fasting FVIIa and in these patients were reduced by treatment with atorvastatin plus placebo. The relationship between eating and acute episodes of VTE is less established, and to date only a single case had been reported in the literature, of a 60 years-old man who was suffering of dizziness during the meals, and after a check-up was verified a cerebral venous thrombosis (Fukutake *et al.,* 2001). The ingestion of fatty meals as a part of daily living mighty lead endothelial dysfunction, hypercoagulability, and platelet hyperactivity that might finally predispose to the onset of acute episodes of thrombosis (Miller, 1998; Anderson *et al.,* 2001). Fututake and coworkers (2001) also speculated that episodic congestion of the jugular venous drainage during mealtime due to an increase in the circulatory volume of the external carotid-jugular system might have contributed to trigger thrombosis. The apparent role suggested by those studies on the etiology of thrombosis was contradicted by a longitudinal approach 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 (Kostapanos *et al.,* 2010). Our hypothesis is that the APOA1 can be a protective protein released by the organism in critical situations, in order to minimize the damage. This hypothesis is corroborated by the study of Jin and coworkers (1998), which was performed trying to better understand the mechanism involved in the reduction of the cardiovascular risk of postmenopausal women under estrogen therapy. They verified that the estrogen therapy increases plasma HDL levels, triggers the accumulation of apoA-I in the stimulated Hep G2 media and stimulates newly transcribed hepatic apoA-I mRNA without effect on apoA-I mRNA half-life.

APOA1BP also would bind to APOA2 and high-density lipoprotein (HDL) in a yeast two-hybrid screen (Ritter *et al.,* 2002; Kalbitzer *et al.,* 2007). The work of Kula and colleagues (2008) suggested an enzymatic function for APOA1BP that is likely to include a nucleotide-containing substrate during capacitation at the level of cholesterol efflux that may be regulated by phosphorylation. Thus, the genetic loci associated with hyperlipidemia in both mice and men include the site of the APOA1BP gene, further supporting the hypothesis that APOA1BP is involved in the process of cholesterol efflux (Jha *et al.,* 2008).

When cells derived from kidney proximal tubules were stimulated with APOA1 or HDL, APOA1BP showed a concentration-dependent release into the media implicating its role in 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.

Another interesting protein found was the **sorbitol desidrogenase (SORD).** In the polyol pathway, the aldose reductase (AR) is the first and rate-limiting enzyme, and promotes the reduction of glucose to sorbitol with NADPH as the cofactor. The second enzyme in this via is the SORD, responsible for oxidation of sorbitol to fructose using NAD(+) cofactor. Carr and Markham (1995) stated that the accumulation and toxicity of sorbitol in specific tissues had been implicated in the development of microvascular problems in some diabetic patients. Recently, the sorbitol via had been implicated in osmotic and oxidative stress (Lo *et al.,* 2007). The SORD production is favored by low oxygen conditions, and is an alternative way to the glucose oxidative metabolism.

Three very similar studies were performed in order to evaluate the effects of inhibition AR or SORD over the cerebral ischemic injury (Lo *et al.,* 2007) and ischemia-reperfusion (I/R) induced myocardial infarction (Tang *et al.,* 2008) in animal models. In the first study, the researchers noticed that the AR deletion protected animals from severe neurological deficits and large infarct. AR(-/-) brains showed lower expression of transferrin and transferrin receptor with less iron deposition and nitrotyrosine accumulation. Most interestingly, a pharmacological inhibition of AR by Fidarestat also protected animals against cerebral ischemic injury. Tang and collegues (2008) found that inhibition of AR or SORD both attenuated the I/R-induced myocardial infarction by the increase of hypoxia inducible factor-1 alpha, transferrin and its receptor, and intracellular iron content. The inhibition also reduced the I/R-induced infarct area of the heart. They also found that the polyol pathway activity could increase the cytosolic NADH/NAD+ ratio, and consequently activate the hypoxia inducible factor-1 alpha, which would induce the expression of transferrin receptor, which in turn would increase transferrin uptake and iron accumulation and exacerbates oxidative damage. This was confirmed by the fact that administration of the iron chelator deferoxamine attenuated the I/R-induced myocardial infarction. Ananthakrishnan and coworkers (2011) determined a significantly higher expression and activities of AR and SORD in hearts from aged rats subjected to global ischemia followed by reperfusion, and the induction of ischemia further increased AR and SORD activity in the aged hearts. Blockade of AR reduced ischemic injury and improved cardiac functional recovery on reperfusion in aged hearts. Apparently, this protein would be foreseen as a consequence of hypoxia derived on an acute DVT episode. However, it was markedly preset on recurrent DVT, indicating that these patients probably present an oxygen privation, as occur in post-thrombotic syndrome.

Summarizing, a diverse group of proteins associated with inflammatory status and metabolic alteration in patients presenting DVT were characterized in this study. We can speculate that these proteins could represent harmful and protective proteins as an attempt to equilibrate the damage. New direct experimental studies evaluating the role of these proteins could reveal more pitfalls on the maintenance of the thrombogenic status.

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CAPÍTULO 2

"Inflammatory, Immune And Lipid Transportation Proteins Are Differently Expressed In Spontaneous And Proximal Deep Vein Thrombosis Patients"

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INFLAMMATORY, IMMUNE AND LIPID TRANSPORTATION PROTEINS ARE DIFFERENTLY EXPRESSED IN SPONTANEOUS AND PROXIMAL DEEP VEIN THROMBOSIS PATIENTS

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Key words: Deep Vein Thrombosis, Proteomics, Plasma, Inflammation, Immune System, Lipid Transportation

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Abbreviations: VTE: Venous Thromboembolism; PE: Pulmonary Embolism; DVT: Deep Vein Thrombosis; MPS: Microparticles; PPP: Platelet Poor Plasma; DTT: Dithiothreitol; ACN: Acetonitrile; TFA: Trifluoroacetic Acid; SCX: Cation Exchange; UPLC: Ultra

Performance Liquid Chromatography; RP-LC: Reverse Phase Liquid Chromatography; ESI Q-TOF: LTQ: Eletrospray Quadrupole Time-of-Flight; SDS: Sodium Dodecyl Sulfate; ITI: Inter-Alpha-Trypsin Inhibitor; IHRP: ITI Family Heavy Chain Related Protein; IaIp: Inter-Alpha Inhibitor Proteins; CRP: C-Reactive Protein; IL-6: Interleukin-6; SAPS: Secondary Antiphospholipid Syndrome; PAPS: Primary Antiphospholipid Syndrome; SLE: Systemic Lupus Erythematosus; aPL: Antiphospholipid Antibodies AT: Artherial Thrombosis; MI: Myocardial Infarction

SUMMARY

Deep vein thrombosis (DVT) is multi-causal disease associated to a high morbidity and mortality due to complications as pulmonary embolism and post-thrombotic syndrome, and around 25 % of the patients will present recurrence in 5 years. The identification of new factors involved in the pathophysiology of DVT can help in the management of these patients, prevention of recurrence and in the development of new therapies. The evaluation of plasma components using proteomics potentially provides a window into the individual's state of health and diseases. Here we analyzed the protein profile of plasma samples from 3 DVT patients and compared to results obtained from 1 sibling and 1 neighbor from each patient. These patients were selected because they presented a personal and family history of spontaneous and recurrent episodes of proximal DVT. METHODS: the albumin was removed using Affi-Gel Blue Gel, and the proteins were alkylated, reduced, precipitated and hydrolyzed by trypsin. The peptides were fractionated by SCX chromatography, and the 7 fractions obtained were directed to the ESI Q-TOF Premier mass spectrometer. The proteins search was performed using Mascot engine against IPI human database. RESULTS: Proteins that were statistically overexpressed in DVT patients included: C4-A

plasma protease, C1 inhibitor Inter-alpha-trypsin, inhibitor heavy chain H1 and the serum amyloid A. Proteins that were statistically reduced in DVT patients included: alpha-2-HS-glycoprotein, isoform 2 of inter-alpha-trypsin, apolipoprotein A-IV and the inhibitor heavy chain H4. CONCLUSIONS: The evaluation of plasma samples from patients with spontaneous DVT allows the identification of proteins that are differently expressed when compared to controls, which maybe have pathological importance for immune and inflammatory processes in DVT.

INTRODUCTION

The thrombosis is the pathologic process of clot formation inside venous and arterial vessels resulted from disequilibrium between the procoagulant, anticoagulant and fibrinolytic activity. Venous thromboembolism (VTE) is considered a common disease, with an incidence from 1 to 3 cases per 1000 individuals (Silverstein *et al.,* 1998; Hanson *et al.,* 1997). A total of 30 % of patients suffering from VTE die within 30 days, a fifth from sudden death due to PE, and nearly 25 % develop recurrent VTE within 5 years (Prandoni *et al.,* 2007). Many acquired and hereditary risk factors for VTE have been described, but in around 25 % of the cases, the predisposing factors remain unknown (Heit, 2002). The identification of new factors associated with deep vein thrombosis (DVT) can result in important insights about prevention, diagnostic and treatment options for these patients.

Traditional coagulation tests, especially clot-based assays, are useful for describing major abnormalities on the hemostatic response, but fail in their application of assessing thrombotic risk on the healthy populations. Recent evidences also attest that the analysis of a vast array of genes can only explain a part of the individual thrombotic risk (Polkinghorne *et al.,* 2009). Human blood plasma can be easily obtained, and contains a combination of proteins, or sub-proteomes, derived from different tissues. Therefore, the evaluation of the protein profiles of human plasma using proteomics methodologies provides an interesting window into an individual's state of health and disease. In fact, these methodologies have already been used in DVT patients (Gelfi *et al.,* 2004; Lippi *et al.,* 2007; Ganesh *et al.,* 2007).

Here, we present a comprehensive proteomic profile of human plasma samples from patients with spontaneous proximal DVT, for whom predisposing factors were not identified.

PATIENTS AND METHODS

Patients

Patients were recruited from the Outpatient Clinic of the Hematology and Hemotherapy Center of University of Campinas – UNICAMP. Samples were collected between February and September 2009. The diagnosis of DVT was confirmed by Doppler ultrasonography. Three patients were selected, all with a history of spontaneous proximal DVT, and without known hereditary thrombophilia, or the diagnosis of any other systemic disease judged by the investigators to have any influence on the plasma proteomic profile of the patiets. A history of VTE in first and second-degree relatives was present in all three patients. 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 over the proteic expression, two control groups composed by siblings and individuals originated from the same geographic area were included. The neighbors had no personal or familial history of DVT, and were matched by gender, age (+/- 5 years), predominant ethnic origin and tobaccoism. 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 preparation

Blood (10 mL) was drawn from the antecubital vein of individuals who did not take any antiplatelet or anti-inflammatory drugs during the two weeks prior to blood collection. Samples were collected into two vacuum tubes (Greiner Bio-One®, Kremsmunster, Austria) containing EDTA. The first tube was used for a complete blood count, so as to avoid the contamination with collagen and thrombin in the proteomic analysis. The second one remained in ice bath until the separation of the platelet poor plasma (PPP) at 4 °C. PPP aliquots were then stored at -80 °C. After that, 250 µL of plasma samples were filtered in 0.45 nm filters (Millipore®, Billerica, MA, USA), and the albumin was depleted with the DEAE Affi-Gel Blue Gel (Bio-Rad®, Hercules, CA, USA) according to the manufacturer instructions. The protein concentration of the albumin-depleted plasma was measured by the Bradford assay. At the time of analysis, the albumin-depleted plasma samples were taken out of -80 °C and put on ice. A mixture of 20 mM DTT in a 100mM Tris-HCl buffer, 12 µL of protease inhibitor (P8465, Sigma-Aldrich®, St. Louis, MO, EUA) and 350 µL of plasmas samples were sonicated and boiled for 5 min. Iodoacetamide (final concentration 50 mM) was added and this mixtures were let sit at room temperature on the dark for 30 min. After that, 5 mL of acetone were added, the sample was vortexed for 5 min and left at

-20 ºC for 2 hours. The samples were taken out of the -20 ºC and were spined at 1500g at 5 ºC for 5 min. The supernatants were discarded and the process was repeated twice using 5 mL of acetone 80 %. The proteins were immediately directed to the digestion process.

Protein *in-solution* digestion

The protein digestion was performed according to Wojcechowskyj and coworkers (2011). The pellets were ressuspended in 300 μL trypsin mix, composed by 0.9 mg of Porcine Trypsin (Promega®, Madison, WI, USA) to 80μg of plasma proteins plus 0.1 % Rapigest (Waters®, Milford, MA, USA in 30 mM NH_4HCO_3), and were let rotate at room temperature for 1 hour. After that, they were put at 37° C overnight. The digestion was stopped with formic acid (2.5 % final concentration) and centrifuged at 20,000 x g for 20 min. The samples were desalted using Sep-Pak columns (C18, Waters®, Milford, MA, USA) and dried.

Peptides fractionation (cation exchange)

The mixture of peptides were fractionated by strong cation exchange chromatography (SCX) applying a PolySulfoethyl ATM column (PolyLC Inc.) coupled to a HPLC Waters 2796 Bioseparations Module and Waters 2996 Photodiode Array Detector. The following gradient was employed for peptide elution at 0.5 ml/min of a KCl buffer (1 mM to 40mM for 8 min), 1M KCL for 2 min, and re-equilibration of the column with 1 M KH_2PO_4 for the last 1,3 min. Eluting peptides were monitored at 214 nm. The peptides were then desalted by a reverse phase applying a Bio Suite C18 PA-A, 3 μm, 2.1 x 150 mm column (Waters, Milford, MA, USA). Fifty fractions of 1.0 ml were collected from the SCS separation and lyophilized. Fractions were combined in 7 pools.

Mass spectrometric analysis

The enzymatic digestion by a site-specific protease product a mixture of peptides, which are introduced inside the mass spectrometer. Ions of a particular peptide are isolated in an initial step of mass spectrometry and collided with gas to produce daughter ions. These fragments are cataloged to produce a tanden mass spectrum. Identification of each MS/MS as a particular peptide sequence is typically performed by database search algorithms such as Mascot (Tabb *et al.,* 2006).

The mass spectrometric analysis was performed according to Paes Leme and coworkers (2011). In a label free analysis, the resulted peptides were resuspended in 20 µL of formic acid 0.1% and an aliquot of 4.5 µl was separated by C18 (10 cm x id 100 µm, 1.7 µm particle size) RP-nanoUPLC (nanoAcquity, Waters) coupled with a Q-Tof Premier mass spectrometer (Waters) with nanoelectrospray source at a flow rate of 0.6 µl/min. The gradient was 2-90% acetonitrile in 0.1% formic acid over 45 min. The nanoelectrospray voltage was set to 3.5 kV, a cone voltage of 30 V and the source temperature was 100°C. The instrument was operated in the 'top three' mode, in which one MS spectrum is acquired followed by MS/MS of the top three most-intense peaks detected. After MS/MS fragmentation, the ion was placed on exclusion list for 60s.

The raw data files were submitted to Mascot Search Engine (Matrix Science®, London, UK). The searches were performed against the IPI human database v3.79, with a parent tolerance of 0.1 Da, a peptide mass tolerance of 0.1 Da, and one trypsin missed cleavage. Iodoacetamide derivative of cysteine and oxidation of methionine were specified as fixed and variable modifications, respectively. The outputs files of the searches were loaded into Scaffold 3 (Scaffold 3_00_3, Proteome Software® Inc. Portland, OR, USA) and the quantitative value was obtained.

Statistical analysis

The quantitative values, which normalize spectral counts across samples to each protein, were compared between patients with their siblings and neighbors. The fold change was calculated by dividing the average counts for each protein identified, and the amounts higher than 1.5 and lower than 0.5 were considered significant.

RESULTS

Three patients with spontaneous proximal DVT were included: one male and two female, 2 Caucasians and one Afro-descendent, all of them presented familiar history of VTE. All patients presented proximal DVT and the median time between the last DVT event and sample collection was 2 years (range 1 to 4 years). One of the patients had controlled hypertension, treated with amlodipine besylate and atenolol besylate. All patients presented proximal DVT and the median time between the last DVT event and sample collection was 2 years (range 1 to 4 years).

The patients and controls blood count presented normal results, and did not present statistically differences between the analyzed parameters. DEAE Affi-Gel Blue Gel successfully depleted the albumin from the platelet poor plasma with reduction of 77.67 % of the total proteins. The 10 % SDS-PAGE gel showed this non-complete depletion, but it was possible to observe one substantial decrease on albumin after the depletion. A third run was performed with one suspension of the column used during the depletion; the albumin and other proteins that could be unspecifically holded by the column or carried by albumin were observed. Currently, there is no procedure available to get rid of albumin without losses of other proteins (Eriksson *et al.,* 2010).

During the fractionation by SCX, the majority of the peptides were released from the column on the first half of the process, and the fractions with lower amounts of peptides were combined, and a total of 50 fractions were reduced to 7 fractions.

Mass spectrometry

After the spectral search by Mascot, it was possible the proteins identification of each one of the three patients and their respective controls (one sibling and one neighbor). The quantitative value, derived by the spectral count (SC) of each protein, was compared between patients and siblings, and the fold change was calculated. The SC depends on the number of MS/MS spectra mapped to peptides with high confidence, so that optimization for SC also favors optimization for total protein identification (Bantscheff *et al.,* 2007). The signal intensities of all ions detectable by MS can be used for quantitation, increasing the reliability of abundance estimates and also extending the dynamic range of MS-based quantitation (Silva *et al.,* 2005). An example of spectrum obtained is shown on figure 1.

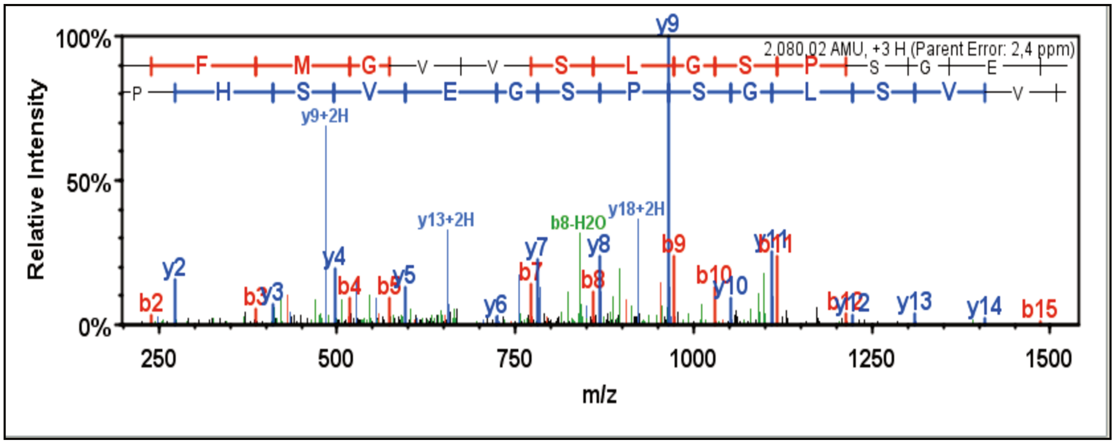

Figure 1. Spectrum of alpha-2-HS-glycoprotein obtained on the label free analysis of plasma samples from Deep Vein Thrombosis patients, their siblings and neighbors.

The number of identified proteins was similar between the groups (199), and the individual analysis of proteins showed significantly higher number of peptides from complement C4-A plasma protease, C1 inhibitor Inter-alpha-trypsin, inhibitor heavy chain H1 and serum amyloid A were detected on patient samples. Peptides from alpha-2-HS-glycoprotein, isoform 2 of inter-alpha-trypsin, apolipoprotein A4 and the inhibitor heavy chain H4 were present in reduced amounts on the patient samples. The results are expressed on table 1.

Table 1. Identification of differentially expressed proteins by LC-MS/MS, showing the accession number, quantitative value (QV) and the fold change presented by the comparison between patients and siblings and patients and neighbors

	Accession number	QV **Patients**	QV **Siblings**	Fold Change	QV **Neighbors**	Fold Change
Complement C4-A plasma protease	IPI00032258	176,99	117,21	1,51	58,42	3,03
C1 inhibitor inter alpha trypsin	IPI00291866	76,86	45,63	1,68	31,51	2,44
Inter alpha trypsin inhibitor heavy chain H1	IPI00292530	9,67	6,37	1,52	3,57	2,71
Serum amyloid A	IPI00552578	12,90	1	12,90	1	12,90
Isoform 2 of inter alpha trypsin H4	IPI00218192	5,00	10,49	0,48	34,41	0,15
Apolipoprotein A-IV	IPI00304273	2,77	10,40	0,27	5,47	0,51
Alpha-2-HS-glycoprotein	IPI00022431	5,95	19,33	0,31	88,66	0,07

DISCUSSION

The DVT is a common disease, and 25 % of patients did not present any risk factors. As the plasma is an important carrier of proteins, and the proteomic tool has a potential to elucidate some factors involved on its pathophysiology.

The depletion of albumin is a crucial part of any plasma proteomic study, due the interference of albumin over the analysis of other proteins. The mechanism by which the albumin interacts with the sepharose contained on the Affi-Gel Blue Gel is not fully clarified. It has been shown that the dye presents affinity for enzymes that utilize AMP for reactions (Bohme *et al*., 1972). Further, it has been suggested that the dye may resemble a nucleotide in shape and thus bind to any protein that has a 'nucleotide fold' (Thompson *et al*., 1975). This tenacious binding of albumin to dye would, on this basis, indicate that it is also capable of binding nucleotides. These hypotheses could explain the high nonspecific bind of other proteins to albumin, and consequently their loss on this analysis. This fact could compromise the results, but all the proteomics studies prefer to assume this bias and many interesting results have been emerged.

In this study, the differently expressed proteins identified between patients and controls seem to play important role on inflammation, immune system and lipid transportation. Although inflammation-induced thrombosis is a well-known mechanism, its pathogenesis is not fully understood. Coagulation also increases inflammation, causing a vicious cycle. This is mainly achieved by thrombin-induced secretion of proinflammatory cytokines and growth factors, and by the triggering of dendritic cells by platelets. There are many systemic inflammatory diseases characterized by thrombotic tendency, including Behçet disease, antineutrophilic cytoplasmic antibody-associated vasculitides, Takayasu arteritis, rheumatoid arthritis, systemic lupus erythematosus (SLE), antiphosholipid syndrome (SAPS), familial Mediterranean fever, thromboangiitis obliterans and inflammatory bowel disease (Johannesdottir *et al.,* 2012)

The C4 complement protein is mainly synthesizes by the liver and it is essential for the propagation of the classical complement pathway (Blanchong *et al.,* 2001). C1s cleaves C4 to C4a and C4b (Schifferli e Paccaud, 1989), and C4A ensures the solubilization of antibody–antigen aggregates through binding to IgG or to antigens of immune complexes (IC), and clearance of IC through binding to complement receptor CR1. Production of complement C4 in excess of the balancing regulatory components could possibly cause over activation of the complement pathways and exacerbate the inflammatory response at the local tissues (Carroll, 1998). Until the moment, there is no specific linking between complement C4-A plasma protease and DVT, but as this protein plays a role on inflammation and the immune system, it has a potential to also participate on DVT physiopathology.

Serum amyloid A (SAA) is a family of acute-phase proteins synthesized primarily by the liver. Plasma levels of SAAs (SAA1 and SAA2) rise dramatically up to 1000-fold in inflammation, as a result of increased transcription by inflammatory cytokines such as TNF, IL-1, and IL-6 (Jensen *et al.,* 1998). This is observed in some inflammatory diseases like metabolic syndrome, diabetes, rheumatoid arthritis or lupus. Indeed, patients with inflammatory disease and DVT, as on SAPS and SLE + antiphospholipid antibodies presented higher SAA, PCR and IL-6 levels. It seems that increased SAA amounts can also indicate a progression from a non-inflammatory thrombotic condition to an inflammatory one (Jensen *et al.,* 1998). On our study, the higher amounts of SAA associated to other proteins also related to inflammation corroborate this hypothesis.

The inter-alpha-trypsin inhibitors (IαI) are a family of structurally related serine protease inhibitors that are mainly secreted by the liver and are present in the blood at considerably high concentrations (0.15–0.5 mg/ml). The main metabolites of IαI-family proteins inhibit a broad spectrum of proteases, including trypsin, chymotrypsin, plasmin, granulocyte elastase, and cathepsin (Chi *et al.,* 2008; Zhuo e Kimata, 2008; Pugia *et al.,* 2007). They seem to be acute-phase proteins, and slightly increased concentrations were observed in plasmas samples from patients with inflammatory disorders (Choi-Miura, 2004). Again, the increased amounts of IαI found in this study also suggest an inflammatory status on DVT patients.

The inhibitors heavy chain H are a family of serine protease composed by five subunits (H1, H2, H3, H4 and H5; Josic *et al.,* 2006), which have been recently implicated in inflammation and cancer metastasis (Fries *et al*., 2003; Lim *et al.,* 2003). Zhang and colleagues (2004) demonstrated an increase of serum subunits H1 and H4 in patients with ovarian cancer when compared to other pathologic pelvic conditions, and supposed that this protein could be considered a marker of this type of neoplasia. In this study, we found increased levels of H1 and decreased amounts of H4 subunits in DVT patients, and further studies are required in order to clarify their relation to the vein thrombosis.

Human α2HS-glycoprotein is synthesized and secreted by the liver, and its concentration decreases significantly following infection (Jethwaney *et al.,* 2005), malignancy (Sakwe *et al.,* 2010), and is regarded as a marker for vascular inflammation and calcification (Baumann *et al*., 2009). Mathews and coworkers (2002) found decreased levels of plasma α2HS-glycoprotein within few hours in MI patients, with return to normal concentration after 5 to 7 days after the acute episode. Bilgir and coworkers (2010) also observed decreased levels of α2HS-glycoprotein in stable angina. Those results indicate that it could be considered a negative acute phase protein in coronary artery disease. We showed that our patients presented decreased amounts of α2HS-glycoprotein, and like in arterial disease, this glycoprotein could be associated to DVT.

The apolipoproteins A1, A4 and E are the main protein constituents of high-density lipoprotein (HDL). It has been established that HDL inhibits the coagulation pathway, decreasing platelet aggregation and thrombus formation (Li *et al.,* 1999; Norfer *et al.,* 1998) probably due direct interactions with platelets (Barlage *et al.,* 2006; Schmitz *et al.,* 2006). Populational studies demonstrate that there is an inverse association between plasma high-density lipoprotein (HDL) levels and recurrent venous thromboembolism (Braekkan *et al*., 2009; Braekkan *et al.,* 2012). Plasma proteomic analysis of patients with Budd-Chiari syndrome showed decreased levels of Apo A1 (Talens *et al.*, 2011). Our results of decreased levels of Apo A4 are interesting and corroborate a participation of those apolipoproteins on the mechanisms of the vein thrombosis.

In this study, only spontaneous and recurrent DVT patients were recruited. Indeed, the strict criteria adopted to select the patients certainly improved the reliability on the results. Besides, the inclusion of two different controls to each patient (one sibling and one neighbor) minimized the genetic and environmental influence on the proteins expression. Our results showed that proteins associated to inflammation, immune system and lipid transportation were differently expressed in patients presenting vein thrombosis. The link between inflammation and DVT has been largely discussed in the literature, and it is corroborated on this study. New direct experimental studies evaluating the role of these proteins could reveal more pitfalls on their involvement on the thrombogenic status.

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DISCUSSÃO GERAL

A TVP resulta de um desequilíbrio entre a atividade procoagulante, anticoagulante e fibrinolítica. O fator tissular, presente em grande quantidade na adventícia dos vasos sanguíneos, exerce um papel fundamental na ativação da coagulação, que ocorre após a injúria vascular. Contudo, não é incomum a trombose ocorrer na ausência de lesão vascular, e assim a ativação seria fruto de outros mecanismos. A complexa combinação entre a predisposição genética e a diversidade fenotípica, composta por fatores adquiridos ao longo da vida do indivíduo somados a fatores desencadeadores torna o estudo desta doença ainda mais desafiador. A busca pelo entendimento de como esta afinada orquestra perde o tom se tornou o nosso mais ambicioso objetivo. Diversas ferramentas podem e devem ser adotadas nesta audaciosa empreitada. Os testes tradicionais da coagulação, principalmente aqueles baseados na formação do coágulo, são imensamente úteis na identificação de grandes anormalidades do sistema hemostático, mas não mimetizam as reais condições em que as reações acontecem no organismo e se mostram falhos na análise do risco trombótico de indivíduos saudáveis.

A descrição do genoma foi o mais importante salto da medicina dos últimos tempos, e forneceu as bases para a uma melhor compreensão de mecanismos fisiopatológicos (Lander *et al.,* 2001; Venter *et al.,* 2001; Waterston *et al.,* 2002). No entanto, a sua transcrição depende da influência de interações bioquímicas associadas ou não com o meio ambiente. As proteínas são consideradas as reais efetoras das reações que promovem os estados de saúde e doença do organismo, e por isso tornam-se alvo na elucidação da fisiopatologia das doenças e na produção de novas drogas, já que mais de 80 % de todos os medicamentos disponíveis agem sobre proteínas (Drews, 2000).

É consensual que as perturbações nas funções proteicas afetam o processo de diversas doenças (Powell *e* Timperman*.,* 2004), e isto poderia estar presente também no processo trombótico. A análise proteômica apresenta um alto potencial em implementar o conhecimento de vias biológicas pois possibilita a identificação de proteínas sub ou superexpressas, além de agregar informações acerca de sua atividade, estabilidade, localização e *turnover*. Exemplos de sucesso no seu emprego foram a identificação de marcadores de neoplasia de bexiga (Ostergaard *et al.,* 1999) e mama (Page *et al.,* 1999).

A relevância das plaquetas na hemostasia é algo estabelecido, e as novas descobertas sobre suas supostas funções sobre o sistema inflamatório e imune foram um grande incentivo para que este estudo fosse realizado. Vários trabalhos têm demonstrado o papel da resposta inflamatória na TVP, mas também na sua relação com a síndrome pós-trombótica (Henke e Wakefield, 2009; Roumen-Klappe *et al.,* 2009; Shbaklo *et al.,* 2009). No entanto, o efeito do trombo nas células que compõem a parede do vaso é pouco compreendido, mas parece resultar em um aumento na rigidez e da espessura da parede vascular, na diminuição da adesão das células endoteliais e em uma matriz com arquitetura aberrante (Henke *et al.,* 2007). É de se esperar que indivíduos com TVP espontânea e de repetição devam apresentar, em níveis individualizados, alterações em proteínas relacionadas à inflamação. O mesmo pode-se dizer sobre o transporte e metabolização de lipídeos.

No estudo do plasma também foi possível a identificação de proteínas intimamente relacionadas à inflamação, imunidade e transporte de lipídeos, embora muitas delas ainda não tenham sido relacionadas à TVP propriamente dita. Novas pesquisas serão imprescindíveis para a determinação se elas só agravariam o quadro de hipercoagulabilidade já presente nestes pacientes ou se teriam poder suficiente para desencadear o processo.

Além disso, alguns comentários se fazem necessários no que se refere ao proteoma do plasma. A depleção da albumina foi uma etapa crítica neste estudo, e três métodos diferentes foram empregados na tentativa de melhor realizar esta tarefa: coluna de afinidade Hitrap Blue HP em FPLC, kit para depleção das 20 proteínas mais abundantes do plasma (ProteoPrep, Sigma) e Affi-gel blue gel. No final, consideramos os resultados deste último como satisfatórios, embora ainda apresentando interferências importantes. Como visto, o seu emprego permitiu a depleção de cerca de 77 % do montante inicial de proteínas, mas através da corrida géis SDS-PAGE pudemos verificar que outras proteínas estavam sendo carreadas neste processo de depleção. O mecanismo pelo qual a albumina é removida do plasma não é esclarecido pelo fabricante, e muito pouco foi publicado até o momento sobre o assunto. Bohme e colaboradores (1972) mencionam que o Cibacron Blue possui uma alta afinidade por enzimas que utilizam AMP em suas reações. Além disso, tem sido sugerido que o corante também possui afinidade a nucleotídeos (Thompson *et al*., 1975). Com isto, estima-se que haja uma grande taxa de ligações inespecíficas durante o procedimento, o que por sua vez pode ter causado a perda de importantes proteínas. No entanto, a análise do plasma se mostra inviável sem que haja a depleção da albumina, tendo em vista que a sua presença ostensiva praticamente impossibilita a análise de proteínas menos expressas, que muito provavelmente possam ter uma grande relevância nos processos patológicos. Assim, o desenvolvimento de técnicas que possam promover a remoção da albumina de maneira específica possibilitaria a análise do plasma com muito mais confiabilidade.

CONCLUSÕES

A análise proteômica mostrou que as proteínas

- sub-unidade ζ1 do coatômero (COPZ1),
- a proteína ligante da apolipoproteina A1 (APOA1BP),
- a hidrolase A4 do leucotrieno (LA4H),
- a desidrogenase 11-17β do estradiol (HSD17β11) e
- a desidrogenase do sorbitol estiveram presentes apenas em plaquetas dos pacientes com TVP, e ausentes nos seus 3 irmãos e 3 vizinhos.

Na análise proteômica de plasmas, verificou-se que as proteínas

- complemento C4-A,
- inhibitor C1 da inter-α-tripsina,
- inibidor H1 de cadeia pesada e
- proteína amilóide sérica A estiveram hiperexpressas, e as proteínas:

- glicoproteína α-2-HS,
- isoforma 2 da inter-α-tripsina,
- apolipoproteína A-IV e o
- inibidor de cadeia pesada H4 estiveram suprimidas nestes mesmos indivíduos.

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ANEXOS

Resumos apresentados em congressos e prêmios recebidos relacionados à tese:

Flores-Nascimento MC, Kleinfelder-Fontanesi K, Orsi FLA, Seeholzer SH, Ischiropoulos H, Annichino-Bizzacchi JM. METABOLIC AND INFLAMMATORY PROTEINS DIFFERENTLY EXPRESSED IN PLATELETS FROM DEEP VENOUS THROMBOSIS PATIENTS. Congresso Brasileiro de Hematologia e Hemoterapia - HEMO, 2011. São Paulo. Categoria: pôster.

Flores-Nascimento MC, Kleinfelder-Fontanesi K, Seeholzer SH, Ischiropoulos H, Annichino-Bizzacchi JM. ANÁLISIS PROTEÓMICO DE LAS PLAQUETAS DE PACIENTES CON TROMBOSIS VENOSA PROFUNDA. XXII Congreso Internacional del Grupo Cooperativo Latinoamericano de Hemostasia y Trombosis. 2011. Montevidéu. Categoria: pôster. Trabalho contemplado com Menção Especial.

Resumos apresentados em congressos não relacionados à tese:

Mazetto BM, Barnabé A, **Flores-Nascimento MC**, de Paula EV, Loureiro de Andrade Orsi F, Annichino-Bizzacchi JM. EVALUACIÓN DE LA ACTIVIDAD DE ADAMTS13 EN PACIENTES CON TROMBOSIS VENOSA PROFUNDA Y SU INTERACCIÓN CON LOS MARCADORES INFLAMATORIOS. XXII Congreso Internacional del Grupo Cooperativo Latinoamericano de Hemostasia y Trombosis. 2011. Montevidéu. Categoria: pôster.

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Nascimento MC; Mazzetto B; Lorand-Metze I; Fenalti Hoehr N; Annichino-Bizzacchi JM. EVALUATION OF CIRCULATING ENDOTHELIAL CELLS IN PATIENTS WITH DEEP VENOUS THROMBOSIS. XXXIII World Congress of the International /society of Hematology, 2010, Jerusalém. XXXIII World Congress of the International /society of Hematology, 2010.

Yokoyama APH; **Flores-Nascimento MC**; Andrade-Orsi FL; de Paula EV; Castro V; Lorand-Metze I, Annichino-Bizzacchi JM. DIAGNÓSTICO DE SÍNDROME DE SCOTT EM PACIENTE COM DISTÚRBIO HEMORRÁGICO DE CAUSA DESCONHECIDA - RELATO DE CASO. In: Congresso Brasileiro de Hematologia e Hemoterapia, 2009, Florianópolis. Revista Brasileira de Hematologia e Hemoterapia.. Rio de Janeiro, 2009. v. 31. p. 62-63.

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Diagnosis of Scott syndrome in patient with bleeding disorder of unknown cause

Mariane C. Flores-Nascimento, Fernanda L.A. Orsi, Ana P. Yokoyama, Fernanda G. Pereira, Irene Lorand-Metze, Erich V. De Paula, Vagner Castro and Joyce M. Annichino-Bizzacchi

Scott syndrome is a rare bleeding disorder due to an impaired exposure of phosphatidilserine on the platelet membrane, compromising the platelet procoagulant activity, thrombin generation and, thus, the clot formation. We report a case of a 17-year-old female adolescent with bleeding episodes of unknown cause. She had normal coagulation, but altered platelet aggregation under arteriolar flow, indicating platelet dysfunction. Furthermore, the expression of Annexin V was markedly reduced and the diagnosis of Scott syndrome was established. She was treated with platelet transfusions and demonstrated a clinical improvement. Scott syndrome may be investigated in cases with bleeding history and normal coagulation tests. ***Blood Coagul Fibrinolysis*** **22:000–000**

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Keywords: Annexin V, bleeding disorder, Scott syndrome

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Introduction

Scott Syndrome is a rare bleeding disorder caused by a less-active phosphatidylserine transporter. Because of this, the phosphatidilserine remains sequestered in the inner leaflet of the platelets, compromising the formation of the tenase and prothrombinase complexes and impairing the thrombin generation, resulting in hemorrhagic complications [1–3]. As this disease is not detectable by standard coagulation tests, further coagulation assays may be necessary for the orientation of diagnosis and laboratory monitoring of the treatment [4].

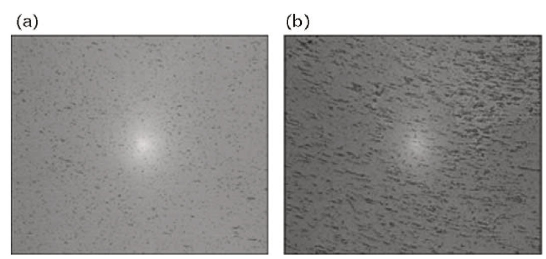
We report the case of a 17-year-old female adolescent with a bleeding disorder of unknown cause, who presented normal specific coagulation tests, but altered platelet aggregation under shear stress.

Case report

A 17-year-old white female adolescent was referred to the Emergency Department of the State University of Campinas in June 2009 to investigate abdominal pain, accompanied by episodes of hematemesis. She also reported other bleeding episodes, such as gingival bleeding, epistaxis, intestinal bleeding due to ulcer, hemorrhoid and hemorrhagic events after three surgeries, which were treated with blood transfusions. During the hospitalization period, she deteriorated clinically, presenting hematuria, hemoptysis, metrorrhagia and severe anemia. The results of the gastrointestinal upper tract endoscopy, pelvic and abdominal ultrasound and chest radiograph were normal and excluded local causes of the bleedings.

Upon hospitalization, the patient presented normal blood counts, despite a mild anemia and normal coagulogram parameters. During the diagnostic investigation, the bleeding time and the plasma levels of the factors V, VII, VIII, X, XI, XIII and von Willebrand factor were normal. Inhibitors of coagulation factors were negative and the plasma levels of fibrinogen were discretely increased (491.1 mg/dl). The platelet aggregation test, applying the agonists 5 μmol/l ADP, 5 μmol/l adrenaline, 2 μg/ml collagen, 0.5 mmol/l arachidonic acid and

Fig. 1



Platelet aggregation under shear stress after applying the Impact: Cone and Plate(let) Analyzer (CPA) Technology. The average size (AS) and area covered (SC) by the platelets aggregates were significantly decreased in the patient (panel a: AS = 21 μm^2, SC = 6.6%) compared with a healthy individual (panel b: AS = 73 μm^2, SC = 11%).

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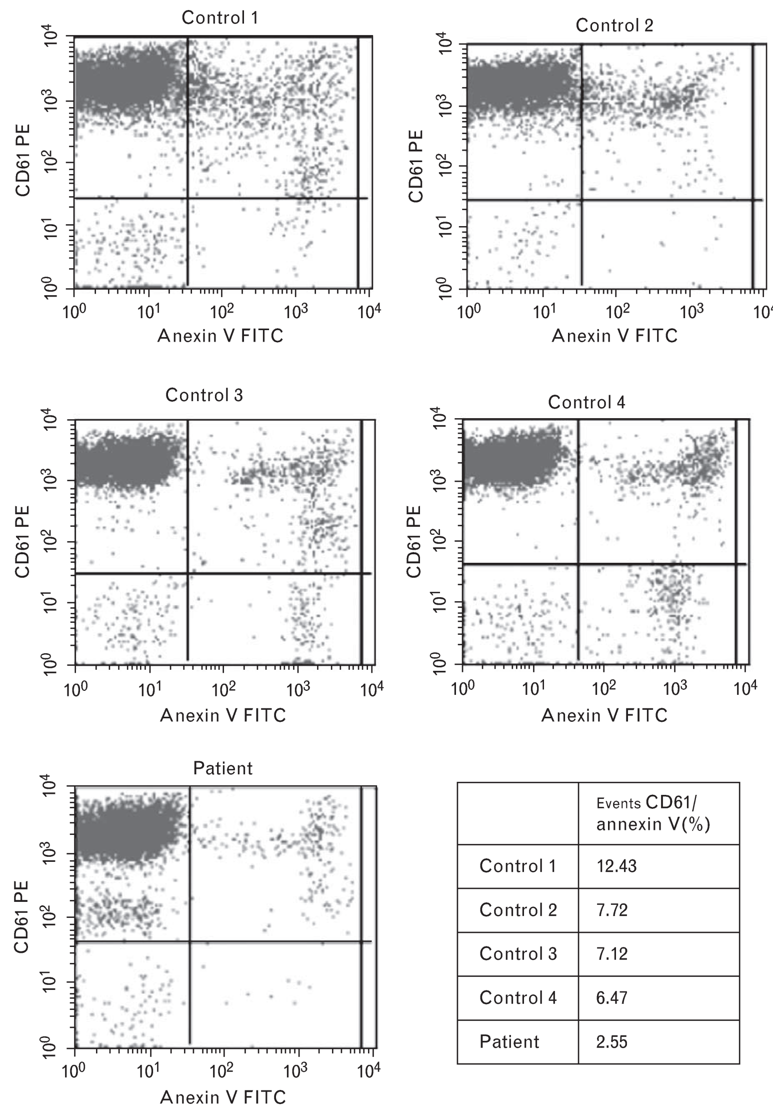
ristocetine (0.6 and 1.25 mg/ml), was also normal. The expression of the platelet surface glycoproteins, GPIb-IX and GPIIb-IIIa was determined by flow cytometry using CD42a, CD42b, CD41 and CD61 monoclonal antibodies and were also normal.

High shear stress platelet aggregation [the Impact: Cone and Plate(let) Analyzer (CPA) Technology] [5] showed decreased platelet aggregate sizes (average size 21 μm^2) and a decreased surface covered by aggregates (area covered 6.6%) in comparison with a healthy individual (Fig. 1a and b; average size 73 μm^2, area covered 11%). This assay confirmed the hypotheses of platelet dysfunction.

The phosphatidilserine exposure on the platelet membrane was then studied by flow cytometry, with thrombin stimulation [6]. Platelet-rich plasma was incubated with

Fig. 2



	Events CD61/ annexin V(%)
Control 1	12.43
Control 2	7.72
Control 3	7.12
Control 4	6.47
Patient	2.55

The phospholipid exposition was analyzed in platelets from healthy individuals (upper plots) and from the patient (lower plot). The platelets were labeled with Annexin V and CD61 without any stimulation (panels a and c) or after incubation with 0.1 U/ml of thrombin with $CaCl_2$ 2.5 mmol/l (panels b and d). On healthy controls, the mean percentage increased from 7.05% before stimulation to 12.27% after stimulation (panel b), whereas the patient's percentage decreased from 6.07% (panel c) to 3.72% (panel d) in the same conditions.

Annexin V-FITC (25 μg/l) for a specific binding to phosphatidilserine and CD61-PE (0.125 g/l) for platelets. The phosphatidilserine membrane expression was two to four times lower on patients' platelets (2.55%) when compared with four healthy controls' platelets (Fig. 2): 12.43% (control 1), 7.72% (control 2), 7.12% (control 3) and 6.47% (control 4).

These results suggest a diagnosis of Scott syndrome. The patient was treated with platelet transfusion, demonstrated an improved clinical outcome and was discharged from hospital 5 days after the beginning of therapy.

Discussion

In the case reported, we present a challenging diagnosis of Scott syndrome. In recent years, there has been a recognition that specific assays of coagulation may not be sufficient to assess the patient's overall haemostatic state, and that more global tests may offer additional information [7]. There is no evidence, however, of which assay should be the standard to evaluate patients with bleeding disorders of unknown cause.

Scott syndrome may be suspected in cases of moderate or severe hemorrhagic disorders associated with normal coagulation tests. Zwaal *et al*. [3] mentioned that platelet count and structure are usually normal, and no aberrations of platelet secretion, aggregation, metabolism, granule content or platelet adhesion to subendothelius have been detected in Scott syndrome carriers.

In the present case, despite the clinical presentation of severe bleeding episodes, all specific tests of coagulation performed gave normal results and only the CPA assay, which evaluates the global function of platelets, raised the possibility of a platelet dysfunction. Recently, this system has been considered a very useful tool for testing platelet function (adhesion and aggregation) under physiological arteriolar flow conditions. This test has been useful for the prediction of bleeding in cardiac surgery patients [8], for the diagnosis of afibrinogenemia, von Willebrand type III, Bernard–Soulier syndrome [9], Glanzmann thrombasthenia and for thrombotic micro-angiopathies, such as thrombotic thrombocytopenic purpura [10]. The CPA assay, in this case, was essential for the diagnosis of platelet dysfunction, and the flow cytometry confirmed the diagnosis, as previously described, of Scott syndrome [6].

Although this is a rare disease, it is important to rule out Scott syndrome in cases of moderate or severe hemorrhagic disorders, associated with normal coagulation tests, in the absence of other compelling explanations. Furthermore, global tests and functional assays of coagulation are required to address the diagnosis of uncommon coagulopathies, frequently diagnosed as bleeding disorders of unknown cause. In this field, CPA assays may play an important role in the diagnosis of platelets disorders.

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Conflicts of interest

There are no conflicts of interest.

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NORMAL LEVELS OF MICROPARTICLES WITH DISCRETE THROMBIN GENERATION CAPACITY IN ACUTE DEEP VENOUS THROMBOSIS

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Key words: acute deep venous thrombosis, microparticles, thrombin generation assay, Annexin V

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SUMMARY

Deep venous thrombosis (DVT) is a multicausal disease, and about 25 % of patients presented spontaneous episodes. Microparticles (MPs) are vesicles released from cellular surfaces during activation and apoptosis, and seems to be increased in hypercoagulability. In a previous characterization of MPs in DVT patients in different conditions applying *in*

house techniques, we did not notice differences on number, cell-origin or procoagulant properties in acute DVT patients. At this time we performed the MPS counting using beads and the procoagulant capacity by the thrombin generation assay in 10 acute DVT patients and 10 healthy controls, matched by gender, age, ethnic origin and tobaccoism. The counting and cell-characterization did not show difference, but the TGA present a short time to peak and an increased area under the curve, although not statistically significant.

INTRODUCTION

Deep venous thrombosis (DVT) is a multicausal disease with several hereditary and acquired risk factors described, but about 25 % of patients presented spontaneous DVT (Miyakis *et al*., 2006). Microparticles (MPs) are vesicles released from cellular surfaces during activation and apoptosis. MPs are present in the circulation of healthy individuals (Berckmans *et al*., 2001), and many authors found increased amounts in situations of vascular damage and hypercoagulability, as disseminated intravascular coagulation (Holme *et al*., 1994), acute coronary syndrome (Katopodis *et al*., 1997), peripheral arterial disease (Zeiger *et al*., 2000), and *diabetes mellitus* (Nomura *et al*., 2003). MPs can induce and propagate coagulation *in vitro* (Ando *et al*., 2002) and can be procoagulant *in vivo* (Biró *et al*., 2003). The presence of MPs in many disorders coincides with an increase of coagulation activation markers, as the prothrombin fragment 1+2.

Many studies have shown an association between high number of circulating MPs and the risk of thromboembolic complications (Mallat *et al*., 2000; Ando *et al*., 2002). In a previous study (Flores-Nascimento *et al*., 2009), we compared the number, cellular origin and procoagulant activity of MPs on DVT patients in different clinical situations: at diagnosis, 1-3 years after warfarin withdrawal, associated to antiphospholipid syndrome, or asymptomatic carriers of Factor V Leiden (FVL) *vs* healthy controls. Our results did not show an increase of the MPs number or the procoagulant activity by an *in-house* thrombin generation test. As those results were unexpected, due the fact that those patients were in a clear status of hypercoagulability, at this time we decided to investigate the DVT patients at diagnosis by standardized techniques, as by the use of count beads and by a commercial kit of thrombin generation.

Patients were recruited from the Emergency Department of the State University of Campinas - UNICAMP. Samples were collected between December 2007 and February 2011. The Local Ethics Committee approved the study and all patients and controls signed a written informed consent. We selected patients with proximal DVT of the lower limbs recruited immediately after diagnosis (n=10, 7F/3M; mean age=43.6; data shown on Table 1). The risk factors for DVT were tobaccoism (n=2), use of hormonal anticoncepcional (n=2), trauma (n=1), and antecedent of DVT (n=1). The diagnosis of DVT was confirmed by Doppler ultrasonography. Patients older than 55 were excluded. For each patient one healthy individual, without personal or familial history of DVT, was matched by gender, age, predominant ethnic origin and use or not of tobacco as controls.

Blood samples were collected into 3.2 % sodium citrate tubes (Vacutainer®, Becton Dickinson, Franklin Lakes, NJ, USA) and the MPs were isolated as previously described (Nieuwland *et al*., 2000). Briefly, blood was centrifuged at 3,000 x g for 20 min, and aliquots of 250 mL of plasma were snap frozen in liquid nitrogen for 15 min, and stored at -80 °C until use. Aliquots were than thawed on melting ice and centrifuged at 13,000 x g for 30 min, and 225 μL of the supernatant were removed. MPs were ressuspended in 225 μL of phosphate-buffered saline (PBS; 154 mmol/L NaCl, 1.4 mmol/L phosphate; pH 7.4) containing 10.5 mmol/L trisodium citrate, and centrifuged under the same conditions. The supernatant (225 μL) was then removed and the resulting pellet containing 25 μL of MPs was used on all the tests.

MPs *characterization by flow cytometry.* MPs were characterized as in our previous study (Flores-Nascimento *et al*., 2009) with modifications. Briefly, samples of MPs suspension (5 mL) were identified using APC-labeled Annexin V (25 mg/L) were also incubated with monoclonal antibodies: platelet (CD61-PE: 0.125 g/L), erythrocyte (CD235-FITC: 0.2 g/L), monocyte (CD14-PE: 0.12 mg/L), leucocyte-derived (CD45-PerCP: 0.125 g/L) and TF-FITC (0.1 g/L at presence with 10 μL of triton X-100). Beads were added in order to quantify the MPs (CountBright, Molecular Probes®, Eugene, OR, USA). The appropriated isotype controls were used, all with 0.1 g/L. The results were expressed as percentage of all events. Surprisingly, no significant differences were detected in the comparison of the total amount or on the cell-specific percentages of MPs from patients and controls (results expressed on table 1).

Thrombin Generation Test (TGA). The determination of the MPs dependent thrombin generation was estimated by the difference between the platelet poor plasma (PPP) and MPs free plasma (MFP) thrombin generation by the Technothrombin MPs kit (Technoclone®, Vienna, Austria) according to the manufacturer instructions. The difference in thrombin generation between PPP and MFP is attributed to the MPs. Briefly, 40 μL of PPP were put in a black microtiterplate with 10 μL of low concentration of phospholipid micelles in Tris-Hepes-NaCl buffer. The thrombin generation was initiated at time 0 by the addition of 50 μL of 1mM fluorogenic substrate Z-G-G-R-AMC with 15 mM $CaCl_2$ and the fluorescence was measured at 60 fixed intervals of 1 min at 360 nm / 460 nm to excitation and emission fluorescence, respectively. After finishing, the experiment was repeated with the MFP, which was obtained by the centrifugation of the PPP at 18,000 x g for 30 min. The supernatant containing the MFP was used in the test. The thrombin concentration was obtained from a reference curve (from 4.2 to 421 nM of thrombin), and all the experiments were performed in duplicate. Many parameters were evaluated, as the lag phase (time to generate 2 nmol/L thrombin; Wielders *et al.,* 2004), the thrombin peak height, the time to the peak and the area under the thrombin generation curve (AUC). No statistically significant increase of the procoagulant activity of MPs measured by TGA test was detected (results shown on table 1), but the time to peak and the area under the curve seemed different between patients and controls.

Statistical analysis The statistical analysis was performed using the GraphPad Instat 3.05 software (GraphPad Software Inc., San Diego, CA) by the application of Mann-Whitney U-test. Outliers showed by patients or controls were excluded from the analysis in order to improve the trustworthiness of the results.

Table 1: Demographic data and results of quantification, cell-derived characterization and thrombin generation assay from acute Deep Venous Thrombosis (DVT) patients and healthy controls.

	DVT patients	**Controls**	**P**
Number	10	10	-
Gender (M/F)	3/7	3/7	-
Age	43.6 (27-54)	41.8 (26-51)	0.80
Number of MPs	424,088 (28,558-1.058,781)	342,291 (9,329-720,799)	0.67
% platelets MPs	66.8 (41.7-94.1)	66.1 (37.4-83.8)	0.78
% erythrocytes MPs	12.1 (3.6-33.1)	16.8 (6.7-37.4)	0.28
% leucocyte MPs	3.1 (0-7.0)	4.7 (1.7-15.4)	0.49
% monocyte MPs	2.4 (0-13.0)	0.6 (0-1.7)	0.37
% TF^+ MPs	23.2 (13.0-29.6)	18.8 (4.4-26.1)	0.39
Lag phase (s)	0.68 (0 – 5.5)	0.63 (0 – 2.5)	0.69
Peak height (nM)	82.2 (18.2 – 160.2)	55.5 (36.1 - 100.6)	0.99
Time to the peak (s)	0.86 (0 – 1.0)	1.63 (0 – 4.0)	0.20
Area under the curve	1,315 (583 – 2,560)	878 (710 – 1,089)	0.89

DISCUSSION

MPs seems to be increased in cardiovascular disorders and other pathological situations in which excessive cell stimulation is present, and their presence in some settings has been correlated with increased coagulation activation (Ando *et al*., 2002; Nieuwland *et al.,* 2000). In our previous study (Flores-Nascimento *et al*., 2009), we already had no difference between the total amounts, percentage or procoagulant signs on the MPs from acute patients, with exception of a D-dimer increase in presence of them. However, in that occasion we did not applied the count beads, but we estimated the MPs by the aspiration of the total volume of all the samples. The thrombin generation test was performed by an *in house* technique, and as our results were contradictory with the literature statements we decided to revisit the same exams with the proper tools. Surprisingly, the results were exactly the same.

MPs are detected and characterized on the basis of antigens characteristic of their respective parental cells (Ahn, 2005). According to this statement, many studies have been performed in order to characterize the MPs in many diseases, including in DVT, but the results are not consistent. While many studies reported the prevalence of PMPs (Chirinos *et al*., 2005; Myers *et al*., 2003), EMP (Smith *et al*., 1999; Musolino *et al*., 1998; Tesselaar *et al*., 2007), monocyte derived MPs (Ye *et al*., 2011) and TF^{+}MPs (Biró *et al*., 2003) in DVT patients, others clearly contradict these statements. Bidot and coworkers (2008) found only a significant elevation of EMPs comparing single episode DVT to controls, but higher amounts of EMP, PMP and RMP in comparison to recurrent DVT patients. Steppich *et al* (2011) measured TF activity, TF antigen, prothrombin fragment F1 + 2, MPs, Interleukin (IL)-1beta, IL-6, IL-8, IL-10, IL-12 and tumor-necrosis-factor-alpha (TNF) and verified that TF antigen, activity and microparticles were similar between 48 acute DVT patients and 45 controls.

The predisposition presented by cancer patients to have a DVT event is well established. It is not unusual the concomitant analysis between DVT patients with and without cancer. To our knowledge, in DVT studies is critical the verification if the included patients have or not this concomitant pathology in order to have clear the etiology of your results. Recently, many studies have been done showing the increased amounts of cell-derived MPs and procoagulant activity in DVT associated to cancer (Tesselaar *et al*., 2009, Zwicker *et al*., 2009, Manly *et al*., 2010). Tesselaar *et al* (2007) found a higher number of MP expressing TF activity in patients with disseminated breast and pancreatic cancer compared to healthy controls, patients with idiopathic acute VTE or non-metastatic cancer. Campello and coworkers (2011) also studied the MPs in cancer patients with and without DVT, and verified higher amounts of circulating EMPs, PMPs, TF^{+}MPs, and the subgroup of cancer patients plus VTE showed statistically significant higher TF^{+}MPs plasma levels than cancer patients without VTE. However, they did not find a significant association between elevated TF^{+}MPs and VTE in cancer patients. Again, the applied criteria for the patients' inclusion in our study were very strict, and we tried to exclude known conditions of hypercoagulability that could interfere with the results, as well as to match the controls. Unfortunately, it was not possible to take the samples before any anticoagulation due the pulmonary embolism prophylaxis therapy adopted on suspicious DVT cases. So, all the patients had at least one dose of low molecular heparin, but we tried to minimize its influence taking the samples right before the next dose.

According to the procoagulant activity, even with no statistical relevance, we can notice a lower time to peak and a higher area under the curve presented by the DVT patients. Maybe the reduced number of patients included could have influenced in the results. Bidot *et al* 2008 evaluated 41 patients with single episode and 25 recurrent DVT patients, and they could observe that MPs-mediated TGA was significantly elevated in patients with DVT compared to healthy controls, and in recurrent compared to single episode patients.

In summary, to us it seems premature the statements about the increased amounts of MPs on DVT. Even with a strong tendency to believe that the MPs could trigger the thrombin generation, it is still premature to postulate a causal role on the thrombus formation. We believe there is a lot of elements playing in this game, and many of them could not been contemplated on the MPs studies. Until now, there is no evaluation method that could minimize the huge distance between the experimental conditions that have been applied and the living ones. Besides, a care inclusion of patients is needed to define if the circulating MPs are sufficient to mediate the thrombus formation.

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