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Biomarkers for post thrombotic syndrome: A case-control study

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ABSTRACT

Introduction: There is limited knowledge on the etiology of post thrombotic syndrome (PTS), although several mechanisms have been proposed.
The objectives are to explore the role of different pathogenic mechanisms for PTS, through measurement of an elaborate panel of biomarkers in patients with and without PTS.
Materials and Methods: Patients with a history of deep vein thrombosis (DVT) with PTS (cases) and without PTS after minimal 2 years follow-up (controls), were selected from the outpatient clinic of two Dutch hospitals. As a reference to the normal population healthy individuals (HI) without a history of venous thromboembolism were invited to participate. The population consisted of: 26 cases, 27 controls, and 26 HI.
A panel of predefined biomarkers was measured in venous blood.
Results: D-dimer showed a decreasing trend from cases to controls to HI; $p = 0.010$. Thrombin/antithrombin complex levels were significantly higher in cases than in controls; $p = 0.032$, and HI; $p = 0.017$. APC-ratio was significantly lower in cases compared to controls; $p = 0.032$, and HI; $p = 0.011$. A significant trend of increasing proTAFI from cases, to controls, and HI; $p = 0.002$ was found. There were no differences in inflammatory markers (CRP, Interleukin-6, Interleukin-8). Thrombomodulin, tissue-plasminogen activator, and von Willebrand factor were higher in patients compared to HI. There was a significant trend of decreasing sVCAM, from cases, to controls, and HI; $p = 0.029$.
Conclusions: Patients with PTS displayed increased coagulation activity, an altered pattern of fibrinolytic marker expression, and increased endothelial activation. We found no evidence of systemic inflammation in patients with PTS at 63 months since the last DVT.

Introduction

Post thrombotic syndrome (PTS) is a prevalent chronic complication of deep vein thrombosis (DVT) affecting 20-50% of patients within the first 2 years after DVT [1–3]. The condition is characterized by complaints of the leg such as pain, heaviness, itching, cramps, and tingling, which may range in seriousness from mild complaints of the leg to intense pain intervening with daily activities. Signs and symptoms of PTS vary over time [1]. In severe cases PTS is accompanied by chronic venous leg ulceration [4,5]. PTS has a significant influence on quality of life and is associated with considerable costs [1,6].

PTS is diagnosed using the Villalta scale, a scoring system developed by Prandoni and colleagues. The Villalta scale consists of 5 subjective complaints (heaviness, pain, cramps, itching, tingling) and 6 objective signs (pretibial edema, skin induration, hyperpigmentation, venous ectasia, redness, pain on calf compression), which are scored from none (0 points) to severe (3 points). Furthermore, the presence of venous ulceration (yes/no) is recorded [7]. The etiology of PTS is not yet entirely understood. Symptoms of PTS are thought to be the end-organ manifestation of venous hypertension, caused by several closely linked processes [8]. Impaired fibrinolysis contributing to persistent (partial) obstruction of veins, increased and continued inflammation, tissue remodelling, and endothelial activation are thought to be involved in the development of PTS [8–11].

Abbreviations: A/C, anticoagulant therapy; APC, activated protein C; CRP, C-reactive protein; DVT, deep vein thrombosis; ELISA, enzyme-linked immunosorbent assay; HI, healthy individuals; Il-6/8, interleukin 6/8; IQR, interquartile range; MMP-9, matrixmetalloprotease 9; n/a, not applicable; PAI-1, tissue-plasminogen activator inhibitor type 1; PAP, plasmin-α-antiplasmin complex; proTAFI, pro thrombin activatable fibrinolysis inhibitor; PTS, post thrombotic syndrome; sICAM-1, soluble intercellular cell adhesion molecule 1; sVCAM-1, soluble vascular cell adhesion molecule 1; TAT, thrombin: antithrombin complex; TM, thrombomodulin; tPA, tissue plasminogen activator; VTE, venous thromboembolism; vWF, von Willebrand factor.

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Upon the occurrence of thrombosis, both thrombus and the venous wall are invaded by leukocytes that secrete growth factors, proteases, and cytokines [8]. The release of proteolytic enzymes and free radicals by leukocytes contributes to damage of the venous valves, resulting in reflux of venous blood [12,13]. Inflammation directs tissue remodelling, by the activation of fibroblasts and smooth muscle cells and deposition of collagen in the thrombosed venous segment, resulting in fibrosis [10].

Comparable to chronic venous disease, continued activation of endothelial cells could also play a role in PTS [13–15]. Attraction and adhesion of leukocytes to sustained activated endothelial cells might be a contributing factor to the chronic inflammation process, with progressive damage of vein wall and venous valves as a result. Matrix metalloproteases (MMPs) have been suggested to be involved in tissue remodelling after venous thrombosis [16,17]. Several studies in mice showed that MMPs play a significant role in the remodelling processes, contributing to post thrombotic venous damage [18,19]. Fibrotic vein walls become stiff and noncompliant and induce increased venous pressure [10].

Thus, the available experimental data suggest a scenario of PTS pathophysiology that involves both prothrombotic and inflammatory mechanisms, linking the blood and vessel wall compartments. Clinically, however, there is very limited data on these processes [9,20]. For this reason we embarked on an exploratory case-control study in patients with and without PTS to assess the potential relevance of a defined panel of biomarkers, reflecting various stages of the postulated pathophysiology of PTS.

Materials and Methods

Patients and Study Design

A case-control study was performed, including 60 patients with a history of objectively confirmed DVT and 30 healthy individuals (HI), without a history of venous thromboembolism (VTE), were invited to participate as a reference to the normal population. HI were spouse, relative, or acquaintance of patients.

Thirty patients with PTS and thirty patients without PTS were selected and recruited from the outpatient clinic of the Maastricht University Medical Centre or the Flevohospital in Almere, the Netherlands.

Patients that had developed PTS (Villalta ≥ 5) were defined as cases. Patients that had not developed PTS (Villalta ≤ 4), after a minimal follow-up of 2 years after DVT, were defined as controls. The majority of patients had visited the outpatient clinic during a period of two years after their DVT, at each visit signs and symptoms were scored according to the Villalta scale. PTS was defined as a Villalta score of ≥ 5 on two consecutive visits, which were at least 3 months apart. When Villalta scores of follow-up were not present, the leg was clinically assessed and a Villalta score was taken at inclusion (6 patients). Patients' legs were assessed by clinicians trained and experienced in scoring legs according to the Villalta scale.

Subjects or patients with chronic inflammatory disease (defined as any manifestation of inflammatory bowel disease or chronic rheumatic disease) or with known venous insufficiency were excluded from the study because of possible interference with the endpoints. Cases, controls, and HI were matched for gender, age, and BMI.

The medical ethical committee of the Maastricht University Medical Centre approved the study and all participants gave written informed consent.

Measurement of Biomarkers

Venous blood was drawn from all subjects and collected in citrate (3,2% w/v) and EDTA polypropylene tubes for plasma (BD Vacutainer), and in clot activator (coating of micronized silica particles) containing polypropylene tubes for serum (BD Vacutainer). Citrate tubes were centrifuged for 5 minutes at 2500 g (3790 rpm, room temperature) and for 10 minutes at 10 000 g (11 000 rpm, 18 °C). EDTA tubes were centrifuged for 5 minutes at 2500 g (3790 rpm, room temperature). Serum tubes were left for 30 minutes at 37 °C and consequently centrifuged for 5 minutes at 2500 g (3790 rpm, room temperature). Plasma and serum samples were stored at -80 °C until analysis.

The panel of biomarkers was based on a systematic review of literature on biomarkers and PTS in humans [20] and extensive review of literature on biomarkers and PTS in animal studies.

A multiplex multi-array electrochemiluminescence platform was used to measure Interleukin-8 (Il-8), Interleukin-6 (Il-6), Matrix metalloproteinase-9 (MMP-9), C-reactive protein (CRP), soluble Intercellular Adhesion Molecule 1 (sICAM-1), soluble Vascular Cell Adhesion Molecule 1 (sVCAM-1), P-selectin, and Thrombomodulin (TM) in EDTA plasma (MesoScaleDiscovery, Gaithersburg, MD, USA) [21]. Von Willebrand factor (vWF) was measured in citrate plasma with a home-made enzyme-linked immunosorbent assay (ELISA) using Polyclonal Rabbit anti Human vWF (DAKO A0082, Glostrup, Denmark) as capture antibody and polyclonal Rabbit anti vWF/HRP (Zebra bioscience P0226, Enschede, the Netherlands) as detection antibody.

Single commercial ELISAs were performed for the measurement of plasminogen activator inhibitor type 1 (PAI-1) (Hyphen Biomed, Neuville-sur-oise, France), thrombin:antithrombin complex (TAT) (Siemens, Marburg, Germany), tissue-plasminogen activator (tPA) (Hyphen Biomed, Neuville-sur-oise, France), plasmin-alpha(2)-antiplasmin (PAP) complexes (Technoclone GmbH, Vienna, Austria), and pro thrombin activatable fibrinolysis inhibitor (proTAFI) antigen (Hyphen Biomed, Neuville-sur-oise, France).

The activated protein C (APC-)ratio was measured with an APTT-based APC resistance assay (Instrumentation Laboratory Company, Bedford, USA). For the used LOT-number an APC-ratio of > 2.39 was defined as not resistant.

D-dimer was measured with the Innovance D-dimer assay (Siemens, Marburg, Germany).

Statistical Analyses

SPSS 21.0 statistical software was used for all statistical analyses (SPSS Inc., Chicago, IL, USA).

Non-parametric Mann Whitney U-tests were performed to test for differences in marker levels between patients (cases and controls) and HI. Subsequently, non-parametric Kruskal-Wallis tests were performed to test for differences in marker levels between the three groups (cases, controls, HI). Post hoc Mann-Whitney U-tests were performed on significant ($p < 0.05$) Kruskal-Wallis test results.

Also, trend analysis (Jonckheere-Terpstra test) was performed, to test for trends in the data.

Marker levels were described as medians and interquartile ranges (25^{th} to 75^{th} percentile).

Additional analyses were performed to correct for possible confounders such as the use of oral anticoagulation (A/C), the use of statins, the use of acetylsalicylic acid, duration of follow-up after most recent DVT, and recurrent VTE.

Results

Patient Characteristics

Ninety subjects were selected, of which 11 were identified as ineligible, because of the presence of inflammatory disease or venous insufficiency. Thus, the total study population consisted of 79 subjects. Fifty three patients that experienced one or more episodes of DVT were included; of those, 26 patients were diagnosed with PTS (cases) and 27 patients did not have a diagnosis of PTS (controls). Furthermore, 26 HI without a history of VTE were included. Median Villalta score (average of 1-4 measurements) was 7 (interquartile range (IQR): 6-10) for the cases and 2 (IQR:1-3) for the controls. Among the cases, PTS was mild