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Full Length Article

Decreased levels of procoagulant phospholipids in bleeding patients treated by vitamin K antagonists

Emmanuel Mathieu [a], Patrick Van Dreden [b], Jérôme Aulagnier [a], Matthieu Grusse [b], Jean-François Dreyfus [c], Dominique François [d], Marc Vasse [e,*]

[a] Emergency Department, Hôpital Foch, Suresnes, France
[b] Diagnostica Stago, Gennevilliers, France
[c] Clinical Research Unit, University of Versailles & Foch, Suresnes, France
[d] Clinical Biology Department, Hôpital Foch, Suresnes, France
[e] Clinical Biology Department, Hôpital Foch, Suresnes, & INSERM UMR_S 1176, Université Paris-Sud, Le Kremlin-Bicêtre, France

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SUMMARY

International Normalized Ratio (INR) is currently used to monitor vitamin K antagonist therapy, and the bleeding incidence becomes exponential for INR > 4.5. Inversely, more than 50% of patients with a supratherapeutic INR are asymptomatic. Therefore it could be of interest to identify patients with a higher bleeding risk. Microparticles derived from different cell types express procoagulant phospholipids (PPL) which can be evaluated by a chronometric coagulation assay where a shortening of the clotting times is associated with increased levels of PPL. In a series of 174 consecutive patients referred to our Emergency Department with an INR > 5, median level of PPL was significantly ($p = 0.004$) lower (38.2 s) in the 119 asymptomatic patients than in patients with nonmajor (43.6 s, $n = 35$) or major bleeding (46.6 s, $n = 19$), indicating higher levels of procoagulant phospholipids in asymptomatic patients. By receiver operating characteristic curve analysis, a cut-off of 43.5 s discriminated patients with higher haemorrhagic risk (area under the curve = 0.648). In contrast, thrombomodulin levels, quantified either by immunological or functional assays were not significantly different between both groups.
In conclusion, evaluation of PPL could be of interest to define the haemorrhagic risk of VKA- treated patients.

1. Introduction

Treatment with vitamin K antagonist (VKA) is widely used for the primary and secondary prevention of thromboembolic events particularly in patients with atrial fibrillation (AF). Even if the use of novel oral anticoagulants (NOA) is increasing, their use in elderly patients is still debated [1] and VKA remain a therapeutic possibility. The increased risk of bleeding in VKA-treated patients is clearly identified, and the risk of major bleeding can be estimated to be around 5% [2]. International Normalized Ratio (INR) is currently used to monitor VKA therapy, and it has been shown that the increase in bleeding incidence becomes exponential for INR values above 4.5 [3] but inversely, more than 50% of patients with a supratherapeutic INR are asymptomatic [4,5]. Therefore it could be of interest to better identify patients with a high bleeding risk. Recently, different scores have been proposed to define high risk patients. HAS-BLED score has been proposed for patients with AF [6]. Its interest in the "real" life led to conflicting conclusions [7,8] and it was thought to overestimate bleeding risk, especially in patients with stage 1 hypertension and/or mild to moderate renal/liver dysfunction [9].

Consequently, identification of plasma markers that identify patients at high risk of bleeding has a great clinical interest. In retrospective and prospective studies, high levels of plasma soluble thrombomodulin (sTM), quantified by immunological assays, were shown to be associated with bleeding complications during warfarin treatment [10,11]. It was hypothesised that this was due to the capacity of sTM to activate Protein C, leading to the proteolysis of factors Va and VIIIa. The measurement of von Willebrand factor led to conflicting results [12,13].

During the last decade, the importance of plasma procoagulant microparticles (MPs) has emerged [14]. These MPs are derived from different origins: endothelium, red blood cells, leukocytes, platelets or apoptotic tissues. High levels of circulating MPs have been associated with various diseases complicated by thrombosis [15,16], but it was also described that asymptomatic patients with severe thrombocytopenia had higher MP levels than patients with a bleeding tendency [17].

Therefore these data prompted us to analyse whether MPs could play a role in bleeding tendency in patients referred to our institution with overcoagulation by VKA (INR > 5). We compared the MPs levels with sTM, using both an immunological assay and a functional assay,

* Corresponding author.
E-mail address: m.vasse@hopital-foch.org (M. Vasse).

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since it was shown that the activity of TM is dependent of the size of the fragments produced [18].

2. Materials and methods

2.1. Patients and controls

The study was performed between March 2013 and October 2014 on the plasma of patients referred to the Emergency Department (ED) of our institution. Blood samples were collected within the first hour after admission, before any treatment administration. Inclusion criteria were a treatment by VKA and an INR > 5. Immediately after INR determination, residual citrated plasma was frozen, 24/24 h, every day of the week. According to the French ethical laws, further biological investigations were performed after receiving the patient or relative's consent, otherwise plasma samples were destroyed (3 cases). The only other exclusion criteria were patients below 18 years. In the asymptomatic group, some patients presented to the ED several times with an INR >5 during the study period. Arbitrarily, only the results of the first admission were considered for the study.

The intensity of bleeding was evaluated according to the scientific and standardization committee of the International Society on Thrombosis and Haemostasis [19]. A bleeding was considered as major in case of fatal bleedings (2 cases, due to intracranial haemorrhages), a symptomatic bleeding in a critical area or organ (intracranial, intraspinal, retroperitoneal, intraarticular and intramuscular with compartment syndrome) or bleeding leading to transfusion of 2 units or more of red blood cells. All other bleeding events not meeting the above criteria were considered as "nonmajor". The intensity of bleeding was evaluated by the patient or relative's interview, a clinical examination by physicians and nurses from the ED, results of the complete blood count and optionally imagery. The intensity was validated by the 2 physicians who took part to this study (EM and JA).

Reference ranges for procoagulant phospholipids (PPL) and TM activity were established with plasma of healthy blood donors provided by the Etablissement Français du Sang (Versailles, France).

2.2. Sample preparation

Blood was collected into citrated tubes (Sarstedt, Nürmbrecht, Germany) containing 3.2% trisodium citrate as anticoagulant in a ratio of nine parts of blood to one part of citrate. Platelet poor plasma (PPP) was prepared by double centrifugation for 20 min at 2000 *g* at room temperature.

2.3. Analytical determinations

Prothrombin time (PT) and INR were measured using ACL 700 analysers (Werfen, Le Pré Saint Gervais, France). PPL was measured using a factor Xa-based coagulation assay, the STA® Procoag-PPL (Diagnostica Stago, Asnières, France). This test is based on the assay described by Exner et al. [20] and consists of the measurement of clotting time, in the presence of calcium, of a system in which the addition of a phospholipid-depleted substrate plasma makes the test dependent on the PPL of the sample being tested. Plasma is depleted of phospholipids using a phospholipase derived from snake venom. Bovine factor Xa triggers the coagulation cascade downstream, thus eliminating the influence of coagulation factors acting upstream. A shortening of the clotting times is associated with increased levels of PPL activity. This test is highly correlated to the count of microparticules expressing phosphatidyl-serine, using flow cytometry or to platelet-derived microparticules [20]. In agreement with these flow cytometric results, a previous study observed a positive correlation between platelet count and PPL determination in healthy subjects [21]. The median PPL level measured in 80 healthy volunteers was 63.9 s (range: 55.2–72.4) with a normal distribution. Inter-assay and intra-assay coefficients of variation for this method were 0.6% and 1.9% respectively.

Antigenic levels of circulating sTM were determined by ELISA (Asserachrom® Thrombomodulin, Diagnostica Stago). Plasma levels of thrombomodulin activity (TMa) were measured by home-made tests. TMa levels were measured on the STA-R analyser (Diagnostica Stago) using a chromogenic assay based on the ability of TMa to activate PC when incubated with thrombin, PC, polybrene and a fibrin polymerisation inhibitor. The activity was monitored with CBS 4246, a substrate of APC at 405 nm. The median TMa level measured in 60 healthy volunteers was 99% (range: 78–118), with a normal distribution. Inter-assay and intra-assay coefficients of variation for this method were 4.2% and 4.8% respectively [21].

2.4. Statistics

The data were analysed using the Medcalc software (Mariakerke, Belgium). Data are presented as median and range. Comparisons of continuous variables between different subgroups were performed using the Kruskal–Wallis nonparametric test. In case of significance ($p < 0.05$), it was followed by a post-hoc Mann–Whitney test. Coefficients of correlation (r) were calculated using the Spearman's rank test. A two-sided p value <0.05 was considered significant. Receiver operating characteristic (ROC) analysis was performed to calculate cut-off values to discriminate bleeding from asymptomatic patients.

3. Results

3.1. Population characteristics

The main characteristics of the populations studied are presented in Table 1. Patients were divided in 3 groups (asymptomatic, "nonmajor" or major bleeding). Nonmajor and major bleedings were present respectively in 35 (20.1%) and 20 (11.5%) cases, 119 patients (68.4%) were asymptomatic. Of the 55 bleeding patients, 13 (23.6%) required red blood cell (RBC) transfusion, prothrombin plasma concentrates were administered to 7 patients, and fresh frozen plasma (in addition to RBC) was infused to 2 patients.

There were no significant differences concerning the age of the patients between the 3 groups ($p = 0.782$, Kruskal–Wallis test).

AF was the main indication of VKA treatment, and the HAS-BLED score was significantly higher in patients with major bleeding than in asymptomatic patients ($p = 0.009$). No patient with major bleeding was treated for deep venous thrombosis or pulmonary embolism. Patients with nonmajor bleeding of the most frequently received fluindione than patients who were asymptomatic, whereas a higher number of patients with warfarin treatment were asymptomatic. A similar trend was observed for patients with major bleeding however it did not reach statistical significance, certainly because of the small size of this subgroup.

Haematuria was the most common clinical sign in patients nonmajor bleeding (42.9%), while gastro-intestinal bleedings were more often observed in patients with major bleeding (45%). A higher frequency of patients with digestive disorders (diarrhoea, vomiting) was identified in patients with nonmajor bleedings.

There were no statistical significant differences between asymptomatic and bleeding patients in term of possible aetiology for overcoagulation, or frequency of therapies such as statins or amiodarone suspected to influence the haemorrhagic tendency of patients with VKA treatment [5]. We also recorded malignancies, since an increased risk of bleeding was identified in patients with cancer patients treated by antivitamin K therapy [22]. Arbitrarily patients were classified in "active" cancer when a cancer was diagnosed within the last 5 years. A trend for a higher frequency of bleeding was observed in patients with cancer, particularly in patients with major bleeding, but it