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# Vitamin K antagonists favourably modulate fibrin clot properties in patients with atrial fibrillation as early as after 3 days of treatment: Relation to coagulation factors and thrombin generation

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ABSTRACT

*Introduction:* Atrial fibrillation (AF) increases the risk of thromboembolism that is reduced by vitamin K antagonists (VKAs). We sought to investigate changes in plasma fibrin clot phenotype at the onset of oral anticoagulation.

*Materials and methods:* Forty consecutive AF patients (aged 45–83 years, $CHA_2DS_2$-VASc score 3.0 ± 1.5) who started therapy with warfarin or acenocoumarol were studied. Plasma fibrin clot permeability ($K_s$), clot lysis time (CLT), along with clotting factors (F), thrombin generation (TG) profiles and protein C (PC) levels were determined on days 3, 5, 7, 28 and 56 ± 1 since the first dose.

*Results:* AF patients had 16% higher median of $K_s$ and 15% lower median of CLT as early as on day 3 of VKA therapy compared with the baseline (both $p < 0.001$), reaching the plateau values on day 7 and 5, respectively. Higher $K_s$ values on days 1 and 3 were found in AF patients with further stable anticoagulation (both $p < 0.05$). Moreover, FIX explained 32% of the total variability in $K_s$. Multivariate analysis adjusted for potential confounders including time as a predictor showed that vitamin K-dependent (VKD) factors, PC and TG parameters were the predictors of $K_s$ (all $p < 0.0001$), while only the lag phase of TG and thrombin peak predicted CLT (both $p < 0.05$) in AF patients. Regression analysis of time-series showed however, that CLT was also predicted by VKD factors and PC (all $p < 0.05$).

*Conclusions:* Plasma fibrin clot properties in AF patients are favourably modified as early as after 3 days of VKA administration, which might contribute to antithrombotic effectiveness.



## 1. Introduction

Atrial fibrillation (AF) predisposes to ischemic stroke and systemic embolization from 2% to above 20% per year [1]. This high stroke risk can be effectively reduced by 65% by using vitamin K antagonists (VKAs) with a target international normalized ratio (INR) value between 2.0 and 3.0 [2]. VKAs reduce the activity of clotting factors (F) II, VII, IX, and X and coagulation inhibitors such as proteins C (PC), S and Z, due to impairment of protein γ-carboxylation leading to diminished thrombin generation (TG) [3]. It is known that lower thrombin concentrations are associated with formation of looser fibrin networks composed of thicker fibres [4].

In anticoagulated AF patients TG profiles are characterized by a large variability despite INRs within the therapeutic range [5]. Both INRs and TG parameters have been shown to be associated with vitamin K-dependent (VKD) factors, especially with FII and FIX [5]. On the other hand, there were no correlations between INR and thrombin-antithrombin complexes measured in blood emerging from skin incisions in AF patients treated with VKA [6].

It is believed that properties of plasma fibrin gels prepared *in vitro* are similar to those formed *in vivo* during thrombotic events. In experimental models, treatment with warfarin is associated with a markedly increased fibrin network porosity, which corresponds to the INR value [7]. Warfarin shortens also plasma clot lysis time but this effect is not associated with prolonged clotting time [8]. It is however unknown when an increase in clot porosity and/or decrease in lysis time occur and which VKA-induced changes in blood coagulation determine alterations in plasma clot structure.

*Abbreviations:* AF, atrial fibrillation; VKAs, vitamin K antagonists; INR, international normalized ratio; F, factor; PC, protein C; TG, thrombin generation; VKD, vitamin K-dependent; AT, antithrombin; OAT, oral anticoagulant therapy; CLT, clot lysis time; ETP, endogenous thrombin potential; $K_s$, fibrin clot permeability; ttPeak, time to peak; TF, tissue factor.

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Plasma fibrin clots composed of compact, highly branched networks with thin fibres, which are less susceptible to lysis, have been reported in patients with thromboembolic events, including myocardial infarction, ischemic stroke and venous thromboembolism [9]. It is unclear whether AF by itself, even in the absence of thromboembolic manifestations, is associated with prothrombotic fibrin clot phenotype, although compelling evidence indicates that AF is related to a prothrombotic state, reflected by elevated TG and fibrinogen concentrations [10,11].

The aim of this study was to investigate changes in fibrin clot permeability and lysability as a function of time during the initiation of anticoagulation with VKA and within its first weeks in AF patients. We also determined clinical and laboratory factors, including the kinetics of TG, that could contribute to VKA-induced alterations of fibrin clot properties.

## 2. Materials and methods

### 2.1. Patients

We enrolled 40 consecutive patients diagnosed with AF. All subjects started or recontinued therapy with warfarin (5–10 mg/d) or acenocoumarol (4–6 mg/d) and were monitored over a 2-month period. Blood was drawn before the first dose of VKA (on the 1st day of the therapy) and at 5 subsequent time-points during the initiation and stabilization phase of anticoagulant treatment (on the 3rd, 5th, 7th, 28th, and 56th day). The exclusion criteria were as follows: any acute illness, known cancer, hepatic or renal insufficiency, diabetes, heart failure (New York Heart Association III or IV), recent thromboembolic event (<3 months), autoimmune disease, known thrombophilia and a current treatment with heparins or new oral anticoagulants. The Jagiellonian University Ethical Committee approved the study and patients provided written informed consent. Data on demographics, cardiovascular risk factors and current treatment were collected from all patients using a standardized questionnaire. Diabetes was defined as a history of diabetes regardless of duration of the disease, a need for hypoglycaemic agents, or fasting glycaemia greater than 7 mmol/l or 126 mg/dl. Coronary artery disease was confirmed angiographically (>50% stenosis in at least 1 major epicardial artery). The diagnosis of stroke was based on the World Health Organization criteria. The CHA2DS2-VASc score was used to assess the risk for stroke and thromboembolism in AF patients [12], while the HAS-BLED score was calculated to evaluate the bleeding risk [13].

### 2.2. Plasma samples

Fasting blood samples were drawn between 8 and 10 a.m. from an antecubital vein with minimal stasis. Plasma samples (9:1 of 3.2% trisodium citrate) were centrifuged (20 min, 2500 g) within 30 minutes of collection, immediately frozen and stored in small aliquots at -80 °C. Creatinine, glucose and blood cell count were assessed by standard automated techniques.

### 2.3. Laboratory measurements

Fibrinogen concentration and prothrombin time (PT)-INR were measured using an automated coagulometer (Behring Coagulation System [BCS], Siemens Healthcare Diagnostics). Plasma levels of FII, FVII, FX (one-stage PT with the use of factor depleted plasmas; Siemens Healthcare Diagnostics, Marburg, Germany), FVIII and FIX (as above; based on activated partial thromboplastin time, Siemens Healthcare Diagnostics) were evaluated using the BCS. Commercial chromogenic assays were also used to measure PC activity (Berichrom Protein C, Siemens Healthcare Diagnostics) and antithrombin activity (antithrombin III, Siemens Healthcare Diagnostics) using the BCS. C-reactive protein was measured by a latex immunoturbidimetric assay (Siemens Healthcare Diagnostics).

### 2.4. Plasma fibrin clot permeability

Clot permeation coefficient ($K_s$) was assessed using a pressure-driven system. $K_s$ was calculated as follows: $K_s = Q \cdot L \cdot \eta / t \cdot A \cdot \Delta p$, where $Q$ is the flow rate in time $t$ (percolated buffer volume collected within 40 minutes), $L$ is the length of a fibrin gel (around 1.6 cm, measured during each experiment), $\eta$ is the viscosity of liquid (0.938 cP at 24 °C), $A$ is the cross-sectional area (0.0615 $cm^2$) and $\Delta p$ is a differential pressure (3922.66 dyne/$cm^2$). Higher $K_s$ values indicate enhanced clot permeability.

To determine tissue factor (TF)-based $K_s$ we used a method previously described with slight modifications [7]. Briefly, 100 μl of citrated plasma were mixed with 20 μl of a reagent mixture at final concentrations of 10 pmol/l TF (Innovin, Siemens Healthcare Diagnostics, Marburg, Germany), 4 μmol/l phospholipids (Rossix, Mölndal, Sweden) and 20 mmol/l $CaCl_2$. The mixture was immediately moved to mechanically scratched tubes (prepared from 1 ml serological pipette, Sarstedt, Nümbrecht, Germany) and incubated for 4 hours in a moisture chamber at room temperature. Later the tubes were connected via a silicone tube to a reservoir containing Tris-buffered saline (TBS, 0.05 mol/l Tris–HCl and 0.1 mol/l NaCl, pH 7.4), with a pressure drop of 4 cm $H_2O$. After washing, flow rates of buffer through the clots were measured. The coefficients of intra- and inter-assay variations were 5.7% and 10.2%, respectively.

### 2.5. Clot lysis time

Fibrinolysis induced by recombinant tPA (rtPA, Actilyse, Boerhinger Ingelheim, Ingelheim, Germany), expressed as CLT, was measured using a slightly modified TF-induced clot lysis assay [14]. Briefly, 85 μl of citrated plasma diluted 1.7 times in assay buffer (25 mmol/l Hepes, 137 mmol/l NaCl, 3.5 mmol/l KCl, 1% bovine serum albumin, pH 7.4) were mixed with 15 μl of a reaction mixture containing $CaCl_2$ at final concentration of 17 mmol/l, diluted recombinant human TF (Innovin, Siemens) at final concentration of 10 pmol/l, phospholipid vesicles (Rossix) at final concentration of 10 μmol/l and rtPA at final concentration of 25 ng/ml. Turbidity was measured at 405 nm and 37 °C. CLT was defined as the time from the midpoint of the baseline clear to maximum turbid transition, to the midpoint of the maximum turbid transition to the final plateau phase. The coefficients of intra- and inter-assay variations were 6.7% and 10.1%, respectively.

### 2.6. Thrombin generation assay

The measurement of calibrated automated thrombogram (CAT) was performed according to the manufacturer's instruction (Thrombinoscope, BV, Maastricht, Netherlands). Briefly, 20 μl of PPP-reagent was added to each well of a microplate containing 80 μl plasma sample. Twenty μl of a fluorogenic substrate, which can be specifically cleaved by thrombin together with $CaCl_2$ (FluCa), was subsequently added to start the reaction. The fluorescence in the plasma was read every 30 seconds during 60 minutes using a Fluoroscan Ascent fluorometer (Fluoroscan Ascent, Thermo Scientific, Vanta, Finland). A curve of the total amount of thrombin generated over time was set up. All samples were tested together with a thrombin calibrator running in parallel. The maximum concentration of thrombin formed during the recording time is described as the "thrombin peak" (peak) and the area under the curve represents the "endogenous thrombin potential" (ETP) [15]. "Lag time" (lag) is the time from the start of analysis until thrombin starts to generate and "time to peak" (ttPeak) is the time from the start of thrombin generation until the maximum thrombin value is achieved [15].

### 2.7. Statistical analysis

Statistical evaluation was carried out using a STATISTICA software (Statsoft Inc, Tulsa, OK). Data were presented as mean and standard

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