

Alterations of the thrombin generation profile in rheumatoid arthritis

Anita Kern^{1,2} · Attila Balog³ · Sonja Dulic³ · Eszter Barabás¹ · Mária Kiszalák² · Barna Vásárhelyi¹

Published online: 19 July 2015

© Springer Science+Business Media New York 2015

Abstract Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disorder resulting in the erosion of the cartilage and bone. Systemic involvement including the cardiovascular system with the risk of atherosclerosis may also occur. Calibrated automated thrombogram (CAT), a commercially available thrombin generation assay is suitable for the general assessment of the functionality of coagulation system. In this study we performed CAT assay in RA patients and in non-affected control subjects (matched for age, sex and comorbidities). Among the CAT parameters Velocity Index increased (from 60 to 83 nM/min), Lag Time and Time to Peak decreased (from 3.47 to 2.83 min and from 6.98 to 5.58 min respectively) in RA. On the other hand, Endogenous Thrombin Potential values decreased (from 1242 to 1108 nM min). The observed alterations were not associated with the applied therapy. These results indicate that the velocity of thrombin formation is increased, while the thrombin generating capability is reduced in RA.

Keywords Thrombin generation · Rheumatoid arthritis · Thrombosis · Biological therapy

Abbreviations

aPTT	Activated partial thromboplastin time
CAT	Calibrated Automated Thrombogram
CRP	C reactive protein
DAS	Disease activity score
DMARD	Disease modifying anti-rheumatic drugs
ESR	Erythrocyte sedimentation rate
ETP	Endogenous thrombin potential
NSAID	Non-steroidal anti-inflammatory drugs
PT	Prothrombin time
RA	Rheumatoid arthritis
RF	Rheumatoid factor
TG	Thrombin generation
TT	Thrombin time
v index	Velocity Index

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disorder with a symmetrical small-joint polyarthritis, but extra-articular manifestations may be present both at disease onset and in established disease. There are a number of studies indicating the augmented risk of cardiovascular diseases in RA including atherothrombosis [1, 2] and venous thromboembolism [3, 4]. However, from the limited data available it is still nebulous whether the increased risk is inherent with RA itself or is partly due to potential confounders [3]. Indeed, in addition to the thrombogenic effects of systemic inflammation, immobilisation, plasma hyperviscosity [5], disturbed venous blood flow [6] and adverse effects of anti-rheumatic agents [7] may also contribute to the thrombotic tendency in RA.

Beside standard coagulation tests [prothrombin time (PT), activated partial thromboplastin time (aPTT),

✉ Anita Kern
kern.anita1@gmail.com; akern@diagon.com

¹ Department of Laboratory Medicine, Faculty of Medicine, Semmelweis University, Nagyvárad tér 4, 1089 Budapest, Hungary

² Diagon Ltd., Baross utca 48-52, 1047 Budapest, Hungary

³ Department of Rheumatology, Faculty of Medicine, Albert Szent-Györgyi Health Center, University of Szeged, Kálvária sgt. 57, 6725 Szeged, Hungary

thrombin time (TT) and fibrinogen] novel biomarkers in the assessment of thrombotic potential have been recently introduced into the laboratory diagnostic arsenal. Thrombin generation (TG) test, a novel global haemostasis assay, is a tool for the general functional assessment of haemostasis. The result of this test reflects reliably thrombotic or bleeding potential that can be triggered by physiologic or iatrogenic stimuli [8]. During TG test coagulation is provoked in the plasma sample by the addition of a small amount of triggering agent (usually thromboplastin). The activity of thrombin is monitored during the entire coagulation process and graphically represented on the thrombogram (Fig. 1). TG profile is characterised by descriptive parameters of the TG curve: Endogenous Thrombin Potential (ETP, total thrombin generating potential of the sample), Peak (maximum thrombin activity), Lag Time (initiation time to thrombin burst), Time to Peak (time to maximum thrombin activity) and Velocity Index (v index, slope of the thrombin burst).

Albeit TG measurements are still not standardized, commercially available tests are already in use. Calibrated automated thrombogram (CAT) is one of the most widely used TG assays. CAT is increasingly used to assess thrombotic risk in a number of disorders including cancer [9], or anticoagulant treatment [10].

Until now only two studies assessed TG in RA. Undas et al. however, collected indirect data regarding TG; they developed an *in silico* model and estimated TG profile by analysing a number of distinct soluble pro- and anticoagulant factors in patients [11]. Their results indicate delayed but augmented thrombin burst in RA samples. However, this study was inherent with several limitations (e.g. the control group consisted of healthy subjects instead of non-affected controls and study population was heterogeneous in term of age and gender). In a more recent study Prati et al. empirically determined the TG profiles in RA [12]. They detected alteration in all TG parameters suggesting a reduced TG in RA (indicative for an increased bleeding

tendency), but no data about the study population and the applied method are provided.

In this study we aimed to evaluate TG parameters in a well-defined and homogeneous RA population and investigated the impact of RA and applied therapy on measured values.

Patients and methods

Patients

Twenty-four and 25 postmenopausal women with and without RA, respectively, matched for age and comorbidities were included in the study. RA patients and control subjects (with joint complaints, but no inflammatory condition) were recruited from the same department. Exclusion criteria were oral anticoagulant therapy or heparin therapy. Patients were treated with corticosteroids (n = 14, 58.3 %), nonsteroidal anti-inflammatory drugs (NSAIDs, n = 5, 20.8 %), biologic agents (n = 10, 41.7 %), disease modifying antirheumatic drugs (DMARDs, n = 22, 91.7 %) and statins (n = 5, 20.8 %). None of the control subjects were treated with corticosteroids, DMARDs and biologic agents. Disease activity score (DAS-28) was calculated for all patients at the time of sampling. Level of standard inflammatory markers [C reactive protein (CRP), erythrocyte sedimentation rate (ESR)] and that of rheumatoid factor (RF) and anti mutated citrullinate vimentin (aMCV) were measured parallel (Table 1).

Patients and subjects gave written informed consent to participate in the study. The study adhered to the tenets of declaration of Helsinki.

Methods

Venous blood samples were drawn in Vacutainer® tubes (Becton–Dickinson, USA) containing 3.2 % sodium

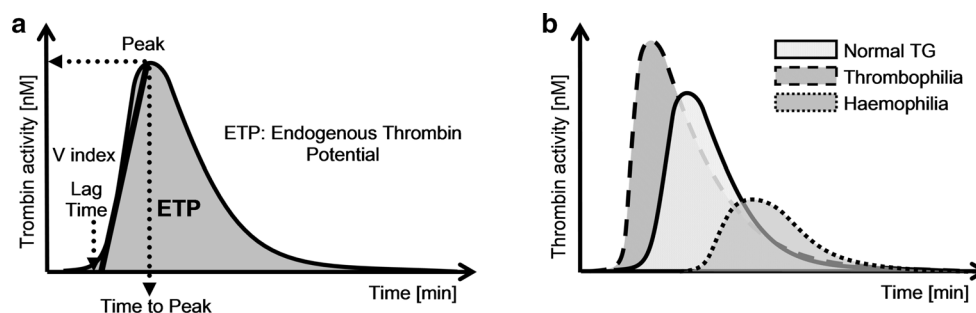


Fig. 1 Thrombogram, graphical representation of a thrombin generation (TG) measurement. **a** Parameters of thrombogram. **b** Characteristic alterations of thrombogram in thrombophilia and haemophilia. Augmented peak, ETP and v index, and decreased Lag Time and

Time to Peak suggest an increase in thrombotic tendency, while reduced peak, ETP and v index, and prolonged Lag Time and Time to Peak indicate an increase in bleeding tendency

Table 1 Clinical characteristics of patients with RA and controls studied

	Patient	Control
Number	24	25
Age (years)	61.7 ± 7.5	56.5 ± 10.8
Duration of RA (years)	11.8 ± 8.8	–
DAS-28	3.29 ± 1.41	–
CRP (mg/l)	5.0 (3.2–11.4) (n = 23)	2.3 (0–4.5)
ESR (mm/h)	26 (12–52) (n = 21)	–
RF (IU/ml)	101.4 (34.9–404.3) (n = 21)	–
aMCV	19 (79.2 %)	–
Diabetes mellitus	3 (12.5 %)	3 (12.0 %)
Hypothyreosis	6 (25.0 %)	4 (16.0 %)
Hypertension	14 (58.3 %)	13 (52.0 %)
Osteoporosis	5 (20.8 %)	5 (20.0 %)
Hypercholesterolemia	6 (25.0 %)	–
Coxarthrosis	–	6 (24.0 %)
Gonarthrosis	–	6 (24.0 %)
Spondylosis	–	15 (60.0 %)

Qualitative data are expressed as number (%) and quantitative data as mean ± standard deviation or median (1st–3rd interquartile), as appropriate

aMCV anti mutated citrullinate vimentin, CRP C reactive protein, DAS-28 disease activity score, ESR erythrocyte sedimentation rate, RA rheumatoid arthritis, RF rheumatoid factor

citrate. For standard coagulation tests (see below) the samples were centrifuged at 2500×g for 10 min. To obtain platelet poor plasma (PPP) for the TG measurement the samples were centrifuged first at 2500×g for 10 min, then at 2500×g for 20 min. Samples were immediately frozen and stored at –80 °C until measurements (done within 1 month).

Standard coagulation parameters (PT, aPTT, TT and fibrinogen) were determined with reagents from Diagon (Hungary) on Coag XL automated coagulometer (Diagon, Hungary).

CAT method (Diagnostica Stago, France) was performed by applying PPP Reagent (Thrombinoscope, The Netherlands). Briefly, 80 µl plasma sample was mixed with 20 µl trigger (PPP Reagent, Thrombinoscope, The Netherlands) in 96 round bottom wells micro plate (Immulon 2HB, Thermo Fisher Scientific, USA). The measurement was started by the addition of 40 µl mixture containing CaCl₂ and fluorogenic substrate (FluCA-kit, Thrombinoscope, The Netherlands). All samples were run in duplicate at 37 °C. Each plasma sample was calibrated individually by applying Thrombin Calibrator (Thrombinoscope, The Netherlands) in order to correct the

fluorescent signal for inner filter effect, substrate consumption and variability in colour of plasma. Fluorescence was measured with Fluoroskan Ascent (Thermo Fisher Scientific, USA) reader applying 390 nm excitation and 460 nm emission filters. TG parameters were calculated with Thrombinoscope Software (Thrombinoscope, The Netherlands). The following TG parameters were determined: Endogenous Thrombin Potential (ETP, nM min), Peak (nM), Lag Time (min), Time to Peak (min) and Velocity Index (v index, nM/min).

Statistical analysis

Normal distribution of data was analysed with Anderson–Darling normality test. Qualitative data are expressed as number (%) and quantitative data as mean ± standard deviation or median (1st–3rd interquartile), as appropriate. Coagulation parameters were compared between RA and non-affected control group, and between RA patients receiving standard, or standard and biological therapy. Comparison between groups was performed with Student t-test in case of normal distribution or with Mann–Whitney U test in case of non-normal distribution. Coagulation parameters of RA patients were correlated with age, disease duration, DAS-28, CRP, ESR and RF. Correlations were assessed with Pearson or Spearman rank coefficient, as appropriate. All tests were two sided, and p values <0.05 were considered statistically significant. Statistical analyses were performed with Minitab® Release 14.1.

Results

Twenty-four patients and 25 control subjects were enrolled into the study. RA patients had significantly higher TT, although the values were within the normal range and the difference was clinically irrelevant. PT, aPTT and fibrinogen levels were not statistically different. On the other hand, several CAT parameters differed significantly between RA and control group; Lag Time, Time to Peak and ETP were lower, while v index were higher in RA than in control group (Table 2).

None of the standard coagulation parameters were associated with age, RA duration, RF, aMCV or ESR values in the RA group. Within RA group, fibrinogen levels correlated with DAS-28 ($r = 0.680$, $p < 0.0005$) and with CRP ($\rho = 0.663$, $p = 0.001$). DAS-28 also correlated with Lag Time ($\rho = 0.438$, $p = 0.032$) and v index ($\rho = 0.526$, $p = 0.008$). No difference was observed in RA subgroups receiving DMARDs and those on combination with DMARD and biologic agent.

Table 2 Comparison of standard coagulation tests and CAT parameters of rheumatoid arthritis (RA) and control group, and that of patients on DMARD—biologic therapy and those receiving DMARDs only

	RA	Control	p RA versus control	Biologic agents+	Biologic agents–	p Biologic agents+ versus biologic agents–
Number	24	25		10	14	
PT (INR)	1.05 ± 0.05	1.06 ± 0.05	ns.	1.05 ± 0.04	1.03 (1.00–1.09)	ns.
aPTT (s)	35.3 (33.9–39.8)	34.3 ± 3.3	ns.	38.1 ± 5.9	34.7 (33.6–36.7)	ns.
TT (s)	18.8 ± 1.2	17.7 ± 1.2	0.003	18.7 ± 1.2	18.8 (18.1–19.2)	ns.
Fibrinogen (g/l)	3.29 ± 0.56	3.09 ± 0.51	ns.	3.13 ± 0.57	3.41 ± 0.55	ns.
ETP (nM min)	1108 ± 168	1242 ± 186	0.012	1157 ± 196	1074 ± 142	ns.
Peak (nM)	236 ± 44	209 ± 60	ns.	234 ± 53	226 (208–238)	ns.
Lag Time (min)	2.83 (2.67–3.25)	3.47 ± 0.91	0.039	2.72 ± 0.33	3.12 ± 0.69	ns.
Time to Peak (min)	5.58 ± 0.79	6.98 ± 1.67	0.001	5.35 ± 0.70	5.74 ± 0.83	ns.
v index (nM/min)	83 (72–121)	60 (35–104)	0.017	98 ± 35	79 (73–106)	ns.

Data with normal distribution are expressed as mean ± standard deviation and in case of non-normal distribution as median (1st–3rd interquartile)

Significant differences are marked with bold letters

aPTT activated partial thromboplastin time, ETP endogenous thrombin potential, INR international normalised ratio, PT prothrombin time, TT thrombin time, v index Velocity Index

Discussion

In this study characteristic alterations were demonstrated in TG parameters of RA patients compared to those in the control subjects. The majority of routine tests of hemostasis (except for fibrinogen level) were normal in RA.

CAT measurement as a global haemostasis assay for the assessment of TG provides a comprehensive tool for the assessment of functional state of the haemostatic system; it reflects the contribution of both pro- and anticoagulant processes to the haemostatic balance. As a result CAT can be considered a more sensitive method than routine coagulation tests to describe the thrombotic state of patients in complex haemostatic alterations such as chronic systemic inflammation in RA.

Among the 5 measured CAT parameters (ETP, Peak, Lag Time, Time to Peak, v index) 4 differed significantly in RA from those in the control group. The characteristics of CAT curve was not clearly specific for thrombophilia or haemophilia, as its parameters shared a mixed pattern: kinetic CAT parameters (i.e. reduced Lag Time and Time to Peak and increased v index) suggested an increase in general thrombotic tendency, while reduced ETP indicated an impaired thrombotic potential. Typical examples for TG curves of a RA patient and a control subject are represented on Fig. 2. The net effect of these changes, however, was an earlier and accelerated formation of reduced amount of thrombin. These changes were associated with RA activity (i.e. DAS-28 scores).

The mutual relationship between inflammation and coagulation is well established. While inflammatory

mechanisms shift haemostatic balance towards increased coagulation, coagulation processes can enhance inflammatory response [reviewed in 5, 13]. Inflammation promotes procoagulation and downregulates anticoagulant processes by increasing platelet count and reactivity, elevating fibrinogen and CRP concentrations, inducing expression of protease activated receptors on endothelium, and tissue factor expression on cell surface of leucocytes. (Indeed, in RA patients the determined fibrinogen levels correlated with CRP levels and DAS-28.) Elevated CRP level promotes interactions of monocytes and endothelial cells [14], and formation of plasminogen activator inhibitor-1 [15] and that of tissue factor [16]. Inflammatory mediators affect anticoagulant pathways by the downregulation of thrombomodulin and endothelial protein C

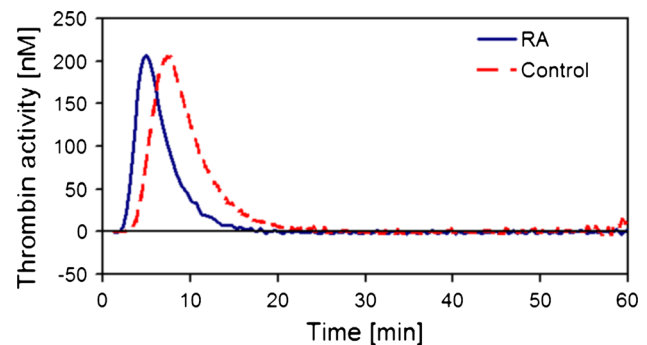


Fig. 2 Typical thrombin generation curve of a rheumatoid arthritis (RA) patient (solid line) and that of a control subject (dashed line). In RA Endogenous Thrombin Potential, Lag Time and Time to Peak is decreased, while Velocity Index is increased

receptor, thus diminishing protein C activation. In inflammation, lowered antithrombin level can be caused by reduced synthesis, degradation and consumption and the decreased synthesis of glycosaminoglycans. Lowered antithrombin activity results in delayed inhibition of the coagulation enzymes that favours thrombotic tendency.

Our observations are not fully in line with the results obtained in a recent study of Undas et al. [11]. This team analysed the TG profile of RA patients calculated with a computational model from their plasma composition. They found that compared to control group RA patients had significantly higher Peak and v index, and prolonged Lag Time and Time to Peak. In contrary to our results, these data indicate delayed but augmented thrombin burst in patient samples.

These contradictions are probably due to different study design, populations tested and methods used. Firstly, there are numerous differences in patient and control group selection. In the previous study the investigated population was heterogeneous in term of age, disease duration and gender, and control group consisted of healthy subjects. Our study focused on the TG profile of postmenopausal RA women, a specific subpopulation, and—to avoid the bias inherent with the use of different age-group—we enrolled age-matched control subjects with a comparable overall health status (without RA). Secondly, Undas et al. determined the TG profile by kinetic modelling, while we measured them empirically. Despite the advances of the kinetic modelling of TG, the limitations of computational modelling should be considered when calculated results are compared to measured parameters [17].

When evaluating the results of our study one should consider that each RA patient participating received DMARD agents combined with or without biological therapy. Theoretically, these agents may also influence directly (through their impact on pro- and anticoagulation factors) and indirectly (through their anti-inflammatory action) the haemostasis. We speculate that these drugs may be responsible that ETP and TT values in our RA patients were inconsistent with kinetic CAT results (they indicated an impairment in TG in RA). The detailed analysis of association between coagulation parameters, CAT parameters and drugs used excluded the contribution of biological therapies to observed alterations. (Literary data support no significant impact of biological agents on thrombotic tendency either [12].)

However, as nearly all RA patients (22/24) were given DMARD for several years, the modulatory effect of DMARD in coagulation system in RA cannot be excluded. Indeed, Undas et al. [11] demonstrated that patients taking methotrexate (a DMARD) had decreased thrombotic tendency. In our population with chronic RA requiring continuous DMARD therapy the possible impact of DMARD agents, therefore, cannot be excluded.

One should also note that our results characterize coagulation in a specific RA subpopulation homogeneous in term of gender (women) and age (postmenopause) and the long term duration of the disease. RA, however, may also affect men and patients of other ages. The possibility that haemostatic conditions in other RA subpopulation are different cannot be excluded and should be the subject for further studies including patients with higher disease activity or patients with early RA.

In conclusion, to our knowledge, we are the first to perform CAT measurements in a homogenous RA population. Our results indicate earlier and accelerated formation of a reduced amount of thrombin. This suggests that RA is associated with alterations of the haemostatic system in a way that it is prepared for fast response to provoking stimuli, thus the haemostatic balance is altered towards thrombotic tendency, but impaired thrombin amount counterbalance this feature.

References

1. Aksu K, Donmez A, Keser G (2012) Inflammation-induced thrombosis: mechanisms, disease associations and management. *Curr Pharm Des* 18:1478–1493
2. Gasparyan AY, Ayvazyan L, Cocco G, Kitas GD (2012) Adverse cardiovascular effects of antirheumatic drugs: implications for clinical practice and research. *Curr Pharm Des* 18:1543–1555
3. Ungprasert P, Srivali N, Spanuchart I, Thongprayoon C, Knight EL (2014) Risk of venous thromboembolism in patients with rheumatoid arthritis: a systematic review and meta-analysis. *Clin Rheumatol* 33:297–304
4. Yusuf HR, Hooper WC, Grosse SD, Parker CS, Boulet SL, Ortel TL (2015) Risk of venous thromboembolism occurrence among adults with selected autoimmune diseases: a study among a U.S. cohort of commercial. *Thromb Res* 135:50–57
5. Hoppe B, Dörner T (2012) Coagulation and the fibrin network in rheumatic disease: a role beyond haemostasis. *Nat Rev Rheumatol* 8:738–746
6. McLaughlin GE (2002) Sudden death in rheumatoid arthritis: pulmonary embolism—a fatal complication of iliopsoas bursitis. *J Clin Rheumatol* 8:208–211
7. Bessissow T, Renard M, Hoffman I, Vermeire S, Rutgeerts P, Van Assche G (2012) Review article: non-malignant haematological complications of anti-tumour necrosis factor alpha therapy. *Aliment Pharmacol Ther* 36:312–323
8. Hemker HC, Al Dieri R, De Smedt E, Béguin S (2006) Thrombin generation, a function test of the haemostatic/thrombotic system. *J Thromb Haemost* 96:553–561
9. Panova-Noeva M, Marchetti M, Spronk HM, Russo L, Diani E, Finazzi G, Salmoiraghi S, Rambaldi A, Barbui T, Ten Cate H, Falanga A (2011) Platelet-induced thrombin generation by the calibrated automated thrombogram assay is increased in patients with essential thrombocythemia and polycythemia vera. *Am J Hematol* 86:337–342
10. Tripodi A, Legnani C, Chantarangkul V, Cosmi B, Palareti G, Mannucci PM (2008) High thrombin generation measured in the presence of thrombomodulin is associated with an increased risk of recurrent venous thromboembolism. *J Thromb Haemost* 6:1327–1333

11. Undas A, Gissel M, Kwasny-Krochin B, Gluszko P, Mann KG, Brummel-Ziedins KE (2010) Thrombin generation in rheumatoid arthritis: dependence on plasma factor composition. *Thromb Haemost* 104:224–230
12. Prati C, Racadot E, Cédoz JP, Toussirot É, Wendling D (2012) Thrombin generation in rheumatoid arthritis. *Joint Bone Spine* 79:522–523
13. Esmon CT (2005) The interactions between inflammation and coagulation. *Br J Haematol* 131:417–430
14. Han KH, Hong KH, Park JH, Ko J, Kang DH, Choi KJ, Hong MK, Park SW, Park SJ (2004) C-reactive protein promotes monocyte chemoattractant protein-1-mediated chemotaxis through upregulating CC chemokine receptor 2 expression in human monocytes. *Circulation* 109:2566–2571
15. Devaraj S, Xu DY, Jialal I (2003) C-reactive protein increases plasminogen activator inhibitor-1 expression and activity in human aortic endothelial cells. *Circulation* 107:398–404
16. Cermak J, Key NS, Bach RR, Balla J, Jacob HS, Vercellotti GM (1993) C-reactive protein induces human peripheral blood monocytes to synthesize tissue factor. *Blood* 82:513–520
17. Hemker HC, Kerdelo S, Kremers RMW (2012) Is there a value in kinetic modeling of thrombin generation? No (unless...). *J Thromb Haemost* 10:1470–1477