

Thromboelastometry, a Possible Tool to Identify Women at Risk of Pregnancy Loss

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Abstract: *Background:* Laboratory methods to study women at risk of fetal loss are essential. Global hemostasis assays are the best methods to detect hypercoagulable states. Thromboelastometry (TEM), may provide extensive information for hypercoagulable states. TEM, performed by the ROTEM (the modified rotation thromboelastogram analyzer), provides a velocity curve of clot formation with new parameters: MaxVel, t-MaxVel, AUC.

Methods: TEM was performed on 87 non-pregnant women (average age 33 years, range 20-45) with a history of recurrent early/or late pregnancy loss and on 76 healthy women (average age 34 years, range 25-42) with no history of pregnancy loss. TEM standard CT, CFT, MCF and ROTEM velocity parameters were assayed. Prothrombin fragment 1+2 (F1+2), Thrombin Activatable Fibrinolysis Inhibitor (TAFI) were also determined. Continuous variables were analyzed using the Mann-Whitney U test. P values less than 0.05 were taken as statistically significant. The correlation between AUC with F1+2 and TAFI values was calculated by the Bland and Altman plot and the Mountain plot methods of comparison.

Results: The CT (51 sec; 49- 59; vs 51sec; 47- 54; p= 0.1113), the CFT (87 sec; 79-95; vs 77 sec; 69- 81; p < 0.0001), the MCF (62 mm; 58- 65; vs 65; 61- 70; p= 0.0020) were not different in women with recurrent fetal loss (RFL) as compared with controls. The MaxVel (14 mm*100 sec; 8-30; vs 15 mm*100 sec; 11-23, p = 0.0989) the t-MaxVel (101sec; 54-146; vs 115 sec; 57-158; p= 0.0649) were not significantly different in women with RFL compared with controls. The AUC (6122 mm; 5074- 6932) was significantly higher amongst patients compared with controls (AUC 5778 mm; range 4683-6784; p < 0.0001). Positive correlation of AUC values was found with higher F1+2 and TAFI levels.

Conclusions: The women with previous fetal loss, tested in our study, showed high AUC values, indicating a hypercoagulable pattern. Therefore, the authors believe that further studies are necessary to clarify whether ROTEM velocity parameters could be utilized to identify women at risk of fatal loss and to suggest a suitable prophylactic regimen to prevent pregnancy loss.

INTRODUCTION

Approximately 1 in every 10 pregnancies ends in early death of the embryo or the fetus and 1 in every 200 pregnancies ends in late fetal loss [1, 2]. A successful pregnancy requires the development of physiological placental circulation. The pregnancy loss (embryonic and fetal loss) may have an impaired placental circulation as the determinant. The inadequate placental circulation (in part due to uteroplacental thrombosis) and/ or abnormal placentation is likely to be influenced by coagulation activation and fibrinolysis at the maternal- fetal interface [1]. Acquired and genetic thrombophilia has been associated with such a condition [3]. Pregnancy is a hypercoagulable state with an increased thrombotic risk throughout gestation and the postpartum period. Women with thrombophilia may have a further increased risk of placental vascular

complications [4]; thrombophilia indeed may increase the risk of placental insufficiency due to thrombosis and also increase the risk of abnormal placentation due to coagulation activation at the maternal-fetal interface [5]. In view of the potential association of thrombophilia and pregnancy loss, and of the high prevalence of thrombophilia in white people, demand for screening has increased [6]. Moreover, as the performance of a comprehensive laboratory screening for thrombophilia is complicated and expensive, new tests for thrombotic risk are eagerly expected. Conventional laboratory clotting techniques can not fully identify subjects with an increased thromboembolic risk. The performance in plasma and the addition of buffered solutions limit their relevance to overall dynamic clot formation in whole blood; in contrast, thromboelastography (TEG), a test on whole-blood hemostasis, specifically assessing overall coagulation, may provide a global evaluation of haemostatic function also in subjects at thrombotic risk [7]. Studies using TEG have previously shown a hypercoagulable state in healthy full term parturients compared to non-pregnant women [8, 9]. Rai *et al.*, have reported that pre-pregnancy MA of TEG was

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predictive of future adverse pregnancy outcome [10]. The same group has shown that serial TEG determinations in early pregnancy, in women with recurrent fetal loss, can identify that increases in the MA precede the pregnancy loss by many weeks [11]. A newer modification of classical thromboelastography is thromboelastometry (TEM). By this technique, the thromboelastographic time course can be transformed into a dynamic velocity profile of the changes in blood elasticity, occurring during clot formation. The software is used to calculate three new parameters in the assessment of coagulation dynamic properties. The pattern of the new values: Max Vel, t- MaxVel, AUC displays a remarkable degree of similarity between endogenous thrombin potential (thrombogram) and the thromboelastometry model [12]. The aims of our study were: 1) to investigate, if thromboelastometry (TEM) may reveal, in women with previous RFL, a pro-thrombotic state that is not identified on conventional tests. 2) to determine whether TEM could be used as a screening test to identify women at risk of early /or late pregnancy loss.

MATERIALS AND METHODS

Patients and Controls

The study was performed in 87 women (average age 33 years, range 20-45) presented to Consultori Familiari of Department Materno-Infantile of Azienda Sanitaria Naples-1 and to the Obstetrics Department at II University of Naples with recurrent early (5-12 weeks) /or late (13-30 weeks) pregnancy losses (65 with early, 22 with late) from 2005 to 2007. All women with 3 or more early fetal loss or with 1 or more late fetal loss were considered for the study. Patients were included after other presumptive aetiological factors of pregnancy failures (chromosomal alterations, endocrine diseases, chronic inflammatory, and infectious diseases) were found to be normal. The patients were excluded from this study if there was a previous history of thrombosis, presence of antiphospholipid antibodies, pregnancy at the time of investigation, or if using oral contraceptives. A group of 76 normal control women (average age 34 years, range 25-42) was also evaluated, who had had only successful

pregnancies. In all cases, blood collection took place at least 12 weeks after their last pregnancy. The study was approved by Ethics Committee of Hospital. Informed consent was collected from all participants.

Samples Collection

Blood samples were drawn between 8 am and 9am, after 12h of fasting by venipuncture from antecubital vein. The blood was collected in a Vacutainer tube containing 0.129 M trisodium citrate for plasma tests performance and TEM analysis. After the TEM study, the blood underwent centrifugation (Megafuge R, Heraeus) at 4500 rpm (3500 g) for 20 min at room temperature; the plasma obtained was separated and stored at -80°C until thrombophilia and coagulation tests were performed. An EDTA whole blood sample was used for PCR analysis of mutations G1691A in Factor V gene, G20210A in the Prothrombin gene, C677T in MTHFR gene

Thromboelastometry

The thromboelastometry was studied on the modified rotation thromboelastogram analyzer (ROTEM[®]; Pentapharm Ltd, Munich, Germany). The ROTEM has overcome some of the limitations of the classical TEG. It is very robust and not susceptible to vibrations or mechanical shocks. By using an electronic pipette, reproducibility and performance has increased. Depending on the parameters measured, ROTEM results are available as early as 15 min up to 1 h. 300 μL of citrated blood were recalcified with 20 μL CaCl_2 0.2M (star-TEM[®] reagent, Pentapharm GmbH, Munich, Germany) and activated by thromboplastin from rabbit brain reagent (ex-TEM[®] reagent, Pentapharm GmbH, Munich, Germany) for monitoring the extrinsic system. The following ROTEM parameters were determined: the onset of the coagulation process: clotting time (CT); the kinetics of clotting formation and stability: clotting formation time (CFT) and maximum clot firmness (MCF) (Fig. 1a).

The continuous coagulation data from a 50 min-time course were transformed into dynamic velocity profiles of WB clot formation. Moreover, the following derivative

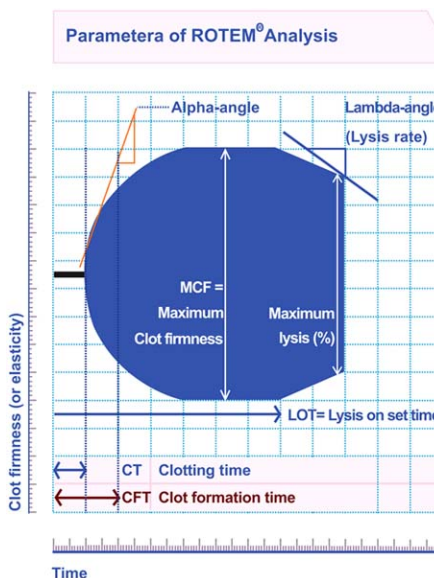


Fig. (1a). Reaction curve and parameters in thromboelastometry.

parameters illustrating the speed of clot initiation and propagation were analyzed: maximum velocity (MaxVel), the maximum rate of whole blood clot formation, the time from start of the measurement till maximum velocity is reached (t- MaxVel), and area under curve (AUC) indicating the maximum clot formation (Fig. 1b) [13].

Prothrombin Fragment 1+2 (F1+2) Measurement

The F1+2 was assayed by an enzyme immunoassay: Enzygnost F1+2 micro, Behring Diagnostica, Marburg Germany [14].

Thrombin Activatable Fibrinolysis Inhibitor (TAFI) Activity Measurement

TAFIa activity was determined by a chromogenic specific 2- step – assay: PEKAFIT TAFI; Pentapharm Ltd, Basel, Switzerland [15]. All the necessary buffers and reagents were prepared according to be manufacturer's instructions. The results are shown as a percentage of normal value.

TAFI Antigen Measurement

TAFI Antigen was assayed by an ELISA test: IMUCLONE TAFI ELISA, American Diagnostica, Stamford. For Italy: IL, Milano [16]. The results were expressed as percentage of normal values.

All women were tested for the presence of Factor V Leiden, prothrombin mutation G20210A, methylenetetrahydrofolate reductase (MTHFR) mutation (C677T), deficiencies of antithrombin, protein C, protein S, APC resistance, according to standard methods [17].

Statistical Analysis

Statistical significance of the differences of values between patients and the controls was calculated by the

Mann- Whitney U test. The differences were considered statistically significant only for p-values less than 0.05. The correlation between thromboelastometric parameters with F1+2 and TAFI values has been analyzed using the Bland and Altman plot and the Mountain plot methods of comparison [18].

RESULTS

TEM standard parameters (CT, CFT, MCF) were not significantly different in women with (RFL) as compared with controls. Amongst ROTEM derivative parameters, AUC was significantly increased in patients as compared with controls (Table 1). F1+2 was significantly higher in patients as compared with controls (Table 1). There were not significant differences in TAFI activity and antigen in patients as compared with controls (Table 1). Nevertheless there was positive correlation of AUC values with F1+2 and TAFI levels: high values of AUC do agree with higher levels of F1+2 and TAFI (Figs. 2-4).

The “**Bland Altman Plot**” [18] assesses the agreement of observed (AUC) and predicted (F1+2) data. The differences between AUC and F1+2 are normally distributed (Gaussian) thence 95% of differences lie between $\pm 2SD$ mean difference.

The “**Bland Altman Plot**” shows that the differences between AUC and TAFI Activities are normally distributed (Gaussian) since 95% of differences lie between $\pm 2SD$ mean difference.

The Altman and Bland (1983; 1986; 1999) test analyses observed and predicted data (the Bland Altman Plot). In order to assess the agreement of observed and predicted data graphically, the difference between the two values (observed and predicted) are plotted against the mean of the two values of the methods for each subject. Limits of agreement,

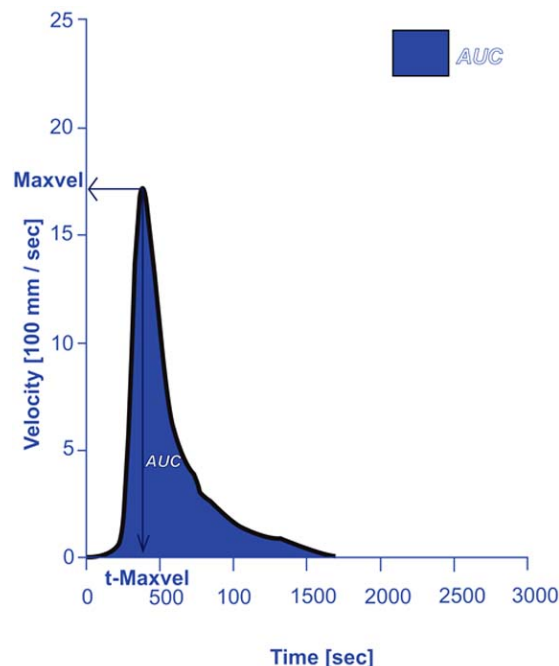


Fig. (1b). The velocity curve and new parameters (MaxVel, t- MaxVel, AUC).

Table 1. Medians and range of CT, CFT, MCF, MaxVel, t-MaxVel, AUC, F1+2, TAFI (Activity and Antigen), in Women (RFL) and Controls

	Women (RFL)	Controls	p-Value
CT (sec)	51 (49 – 59)	51 (47 – 54)	0,1113
CFT (sec)	87 (79 – 95)	77 (69 – 81)	< 0,0001
MCF (mm)	62 (58 – 65)	65 (61 – 70)	0,0020
MaxVel (mm*100/sec)	14 (8-30)	15 (11-23)	0.0989
t-MaxVel (sec)	101 (54-146)	115 (57-158)	0.0649
AUC (mm*100)	6122(5074-6932)	5778(4683-6784)	< 0.0001
F 1+2(ng/ml)	1.01(0.53-1.74)	0.6(0.4-0.9)	< 0.0001
TAFI (activity) %	105(76-147)	110(86-135)	0.1227
TAFI (antigen) ng/ml	112 (62-150)	102 (72-119)	0.0717

defined as twice the standard deviation of the difference between the observed and predicted values, are calculated and plotted in the figure. If we suppose that these differences follow a normal distribution, 95% of the differences will lie between the limits of agreement. If the differences are normally distributed (Gaussian), 95% of the differences will lie between the limits of agreement (or, more precisely, between mean difference $2 \times$ standard deviation [$d^- - 2s$] and mean difference $+ 2 \times$ standard deviation [$d^+ + 2s$]. If the differences fall within the limits of agreement and the limits of agreement are considered to be clinically acceptable in terms of agreement, then one can say the observed and predicted values are in some sense comparable. In the

analysis of measurement method comparison data, neither the correlation coefficient nor techniques such as regression analysis are appropriate. These misleading analyses can be replaced by a “Bland and Altman method” that is simple to do and to interpret [19].

The “**Mountain Plot**” [18] is a useful complementary plot to the Bland and Altman Plot. By this method, it is easier to find the central 95% of the data, even when the data are not normally distributed and different distributions can be compared more easily. The distribution of differences between AUC, F1+2, TAFI Activities shows a positive correlation.

There were no significant differences in incidence of prothrombotic mutations in our women population compared with that of control group. APC resistance with Factor V Leiden was seen in 6 patients (4.92%) compared to 4 controls (4.0%), Prothrombin G20210A was seen in 4 patients (3.2%) compared to 3 controls (3.5%), while MTHFR(C677T) was found in 25 patients (20%) and in 27 controls (30%). We did not find any subjects with antithrombin and protein C deficiencies in either group. Protein S deficiency was present in 3/87, (3.4%) of patients. There was no significant correlation of TEM parameters in those with or without these thrombophilic factors.

DISCUSSION

The hypothesis that recurrent fetal losses are due to a state of hemostatic activation during pregnancy leading to thrombosis of the utero-placental vasculature and subsequent fetal death has been considered since 1980, when the antiphospholipid syndrome was recognized as a cause of recurrent fetal loss [20]. Commonly women with a previous

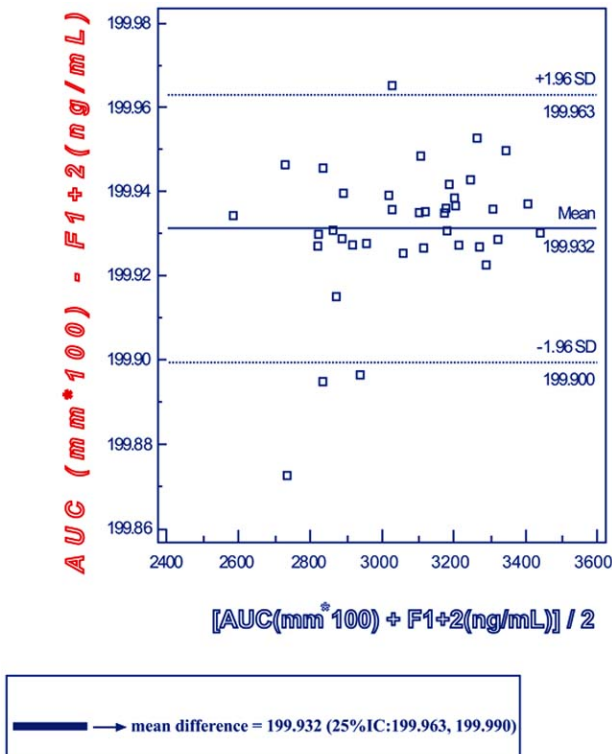


Fig. (2). Correlation of AUC with F1+2.

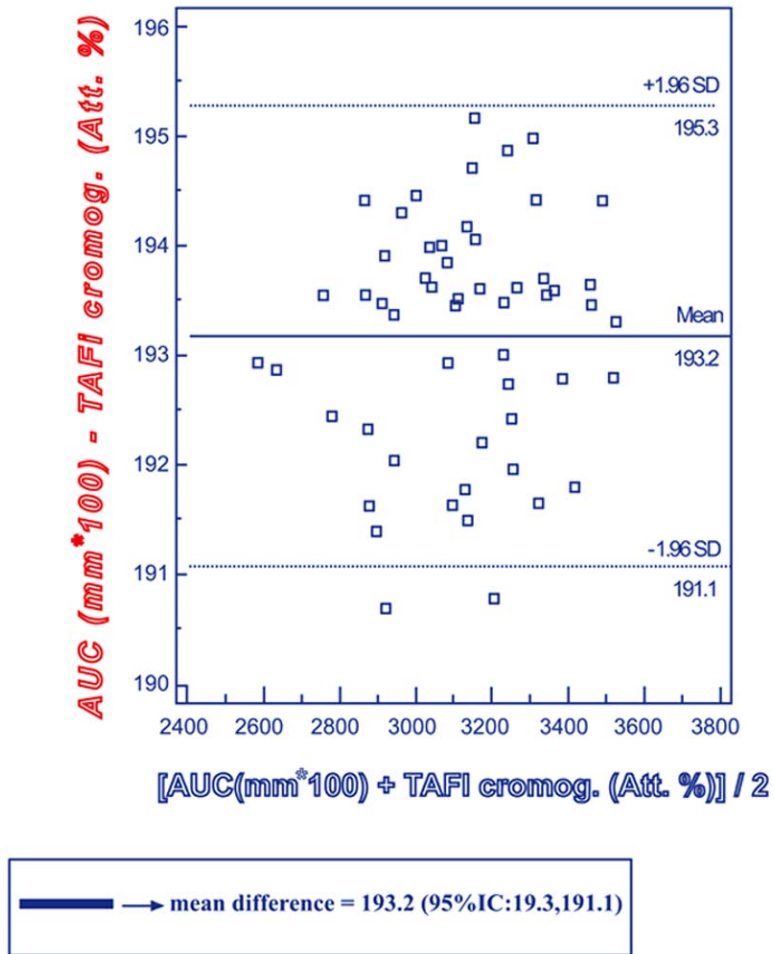


Fig. (3). Correlation of AUC with TAFI activity.

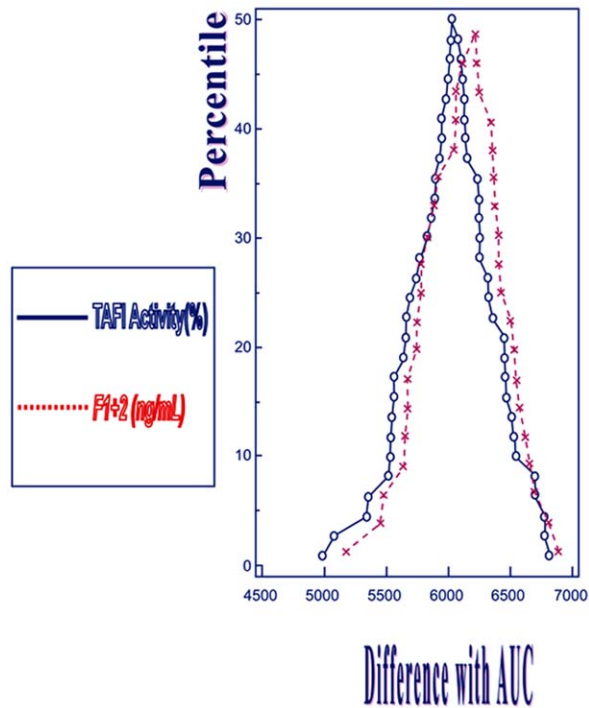


Fig. (4). Correlation between AUC, F1+2 and TAFI activity.

history of pregnancy loss are examined for known thrombophilias but the relative risk associated with a single factor is small and the prognostic significance for an individual is unclear. Thromboelastography is a valuable method which monitors hemostasis under low shear environment, as a whole dynamic process, instead of revealing information on isolated parts of different, linked pathways. Indeed TEG provides information about the inter process of clot formation which results from interdependent steps: coagulation activation, thrombin production, fibrin formation and polymerization, platelet activation, platelet-fibrin interaction. However, TEG may detect hemostatic alterations that lead to a prothrombotic state not revealed on conventional tests. Rai *et al.* have shown TEG to be useful in investigating non-pregnant women with previously unexplained recurrent fetal losses (RFL). The RFL group had significantly greater (MA) compared with control group [9]. Thromboelastometry (TEM), is the latest modification of classical thromboelastography where the activation of the samples accelerates the measurement process and enhances reproducibility. Until now TEM has been utilized mainly to detect hypocoagulable states [21] and to drive therapeutic interventions in patients who undergo major surgery and organ transplantation [22]. To date, it has been rarely utilized in hypercoagulability setting [7, 23]. Therefore, we decided to see whether TEM would be a tool to identify women at risk of RFL. In our study, standard ROTEM parameters, CT, CFT, MCF were not different between patients and controls. Among ROTEM derivative parameters, AUC values, which are expected to better correlate with hypercoagulable state [24], were significantly higher in patients than in controls. An increased level of prothrombin fragment (F1+2), a marker of thrombin generation, amongst non-pregnant women with RFL, complements our study. Increased level of thrombin-antithrombin complexes, in non-pregnant women with previous RFL, compared with controls, was found in the work of Vincent *et al.* [25]. TAFI levels were not increased in women with RFL; nevertheless a positive correlation has been found between high AUC values and higher F1+2 and TAFI levels. The role of TAFI system (proCPU/CPU) is important in the balance between fibrin deposition and removal; the proCPU provides a regulatory link between the coagulation and fibrinolysis. When the system is altered, such as in the case of exacerbated thrombin generation, then, this can lead to an enhanced thrombotic tendency [26]. Therefore TEM, being relatively fast and reproducible, would be a possible tool to study women at risk of fetal loss with or without thrombophilia.

In conclusion, the authors believe that high AUC may indicate a hypercoagulable pattern and further studies should be performed to clarify whether these new ROTEM parameters could be used to identify women at risk of fetal loss. Moreover, to identify as soon as possible a trend towards the hypercoagulable state in women with RFL, without other etiological factors, may be particularly interesting. The antithrombotic treatment in thrombophilic pregnant women with previous RFL has improved prognosis of pregnancy; the possibility of starting early

antithrombotic prophylaxis before the beginning of pregnancy, after a positive TEM analysis, should be considered

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