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Evaluation of the Profile of Thrombin Generation during the Process of Whole Blood Clotting as Assessed by Thromboelastography

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Abstract

Thromboelastography is useful for assessment of whole blood coagulation. The objective of this study was to evaluate the possibility of linking the tracing of whole blood clotted in a thromboelastograph TEG with the generation of thrombin assessed by thrombin/antithrombin complex (TAT). Citrated whole blood containing corn trypsin inhibitor from volunteers was clotted in the presence of $CaCl_2$ and tissue factor. Clotting was monitored with 8 channels of a TEG system. At different time points the whole blood TEG reaction cups were put in a cold quenching solution, centrifuged, and the supernatants were kept at −80°C until assayed for TAT by ELISA. Total Thrombus Generation (TTG) was calculated from the first derivative of the TEG waveform and was compared to thrombin generation measured by TAT. The two vectors of values - the TAT thrombin generation data and the corresponding TEG TTG - were analyzed using Pearson correlation coefficients and linear, non-linear, and natural log (Ln) transformation of TAT values for least-squares goodness-of-fit curves. The best least-squares fit is an exponential curve. Linearizing using the natural log of the TAT thrombin generation variable produces the same R^2 as for the exponential curve. The prediction equation is $y = 8.0465 + 0.0005x$ ($P \leq 0.0001$), where y is the TAT thrombin generation in the Ln transformation variable and x is the TEG TTG variable. The high magnitude of R^2 and the high significance of the prediction equation demonstrate the high efficacy of the prediction of TAT thrombin generation using the TEG TTG.

Keywords

Thrombin generation; Thromboelastograph; Whole blood clotting

INTRODUCTION

Blood coagulation is a complex physiological process which leads to the formation of a fibrin clot through the proteolytic action of thrombin on fibrinogen. In the early days of laboratory assisted clinical practice, the competency of blood for clot formation used to be assessed by whole blood clotting time[1]. With advances in automated technology, clinicians have learned to use tests done on platelet poor plasma with clotting[2] or chromogenic[3] end point. With better understanding of the contribution of platelets, leukocytes and erythrocytes on the process of coagulation, interest has recently focused on the kinetics of thrombin generation in platelet rich

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plasma[4] and in whole blood[5]. The evaluation of thrombin generation during blood coagulation has become the preferred clinically relevant approach to assess the global integrity of this complex process. In order to give the full picture of the physiology of this multifactorial reaction, all cellular and soluble plasma participants to this reaction have to be present in their natural whole blood environment[6]. Whole blood thrombin generation tests are best suited for this purpose but are somewhat cumbersome for use in real time clinical practice.

Thromboelastography is a technology which can assess whole blood coagulation and which was first described more than fifty years ago[7]. It was designed with the intent of evaluating in real time the competency of blood for coagulation, in order to assist in the control of bleeding during surgery. Although many important observations on the physiology of blood coagulation have been made with this technology[8], it has never been widely used, for lack of reliable and easy-to-use instruments. With the advent of microcomputer-assisted equipment, this technology is gaining more and more relevance in clinical assessment of bleeding and thrombotic conditions. This technology has been used extensively in the monitoring of hemostasis during major surgical interventions such as liver transplantations[9,10], cardiovascular procedures[11–15], trauma[16], and neurosurgery[17,18]. It has also been used in the management of obstetrical complications[19–21] and of deep vein thrombosis[22] as well as in the monitoring of antagonists of platelet GPIIb/IIIa[23,24], platelet thromboxane A_2 and ADP[25], and recombinant Factor VIIa[26–27]. Its use has resulted in reduction in the use of blood products[9–12, 15] during surgery, and in the rate of re-exploration in cardiac surgery[15].

The objective of the present study was to evaluate the correlation between the waveform tracing generated by clotting of whole blood in a Thrombelastograph® (TEG®) Haemostasis Analyser and the kinetic profile of thrombin generation, as measured by the reference method of generation of thrombin/antithrombin (TAT) complex. Indeed such a correlation was established.

MATERIAL AND METHODS

Normal subjects

Two subjects, one man and one woman, were studied three times each on three different days. Two other subjects, one man and one woman, were studied once each. TAT complex concentrations (see below) were measured in duplicate and means of each result were used in the different statistical analyses performed.

Materials

HEPES, L-Benzamidine, bovine albumin, EDTA were purchased from Sigma Chemical Co (St. Louis, MO). Innovin was purchased from Dade Behring Inc. (Marburg, Germany). Corn trypsin inhibitor was purchased from Haematologic Technologies Inc. (Essex Junction, VT). D-phenylalanyl-L-prolyl-arginine chloromethylketone was obtained from Cedar Lane Laboratories Ltd. (Hornly, On, Canada). Thrombin/antithrombin ELISA kits were purchased from Dade Behring Inc. (Marburg, Germany). The Thrombelastograph® Haemostasis Analyzers were obtained from Haemoscope Corporation (Niles, IL)

Laboratory testing

Whole blood was obtained by a two-syringe technique and anticoagulated with 3.2% buffered citrate in a 1 citrate: 9 whole blood proportion. Citrate contained 1000 μg/ml of corn trypsin inhibitor, in order to have a final concentration of 100 μg/ml of whole blood. Tubes were capped and kept undisturbed for 30 minutes at 37° C. TEG® reaction cups kept at 37° C were preloaded with 20μL of $CaCl_2$ (200 mM) and 20 μL of Innovin diluted 1/100 in PBS/albumin 4%, pH 7.4. At the time the reaction was started, 320μL of citrated whole blood was added to the cup,

and recording was initiated. Four TEG® units (8 reaction cups) were used simultaneously. At given time points, recording was stopped and the whole reaction cup was quickly dropped into 2640 μL (1/10 final dilution of whole blood clotting mixture) of an ice-cold quenching solution (see below). After vortexing for 10 seconds, the mixture was kept on melting ice until the end of the experiment (about 40 minutes). Specimens were centrifuged at 15,000g for 3 minutes, and supernatants were kept frozen at −80°C until dosage of TAT complexes. The quenching solution used was prepared as follows: 50 mM EDTA, 10 mM L-benzamidine in HEPES 2 mM, NaCl 150 mM, pH 7.4. Immediately before the experiment, 10 μL of 10 mM D-Phe-Pro-Arg chloromethylketone diluted in 0.01 N HCl was added to 2630 μL of the above solution. TAT complexes were measured with the ELISA kit, as recommended by the manufacturer.

TEG® technology

The TEG® system measures whole blood thrombus generation continuously from liquid form to fibrin formation; from thrombus rate strengthening to maximum dynamic property of fibrin-platelet bonding via GPIIb/IIIa, which represents the ultimate strength of the fibrin clot; to eventual thrombus retraction and lysis. This is done by the use of a cylindrical cup that holds the blood as it oscillates through an angle of 4° 45′ (Figure 1).

Each rotation cycle lasts 10 seconds. A pin is suspended in the blood by a torsion wire and is monitored for motion. The torque of the rotating cup is transmitted to the immersed pin only after fibrin and fibrin-platelet bonding have linked the cup and pin together. The strength and rate of formation of these bonds affect the magnitude of the pin motion such that strong clots move the pin directly in phase with cup motion. As the clot retracts and/or is lysed, these bonds are broken and transfer of cup motion is diminished. A mechanical-electrical transducer converts the rotation of the pin into an electrical signal that can be monitored by a computer. The TEG® software produces both graphical and numerical output. A representative signature waveform is shown in Figure 2, and the numerical parameters are described in Table 1.

Thrombus Dynamics

The resultant hemostasis profile can be evaluated and individual points in the profile indicate specific parameters of a patient's hemostasis (Table 1) Figure 3 is a representative TEG® tracing of one of the normal controls. The TEG® waveform was obtained during the clotting process of normal whole blood where coagulation was initiated by recalcification in the presence of diluted Innovin, as described in Methods. The shaded area represents the corresponding first derivative or velocity of the waveform produced by the TEG® system. Several parameters corresponding to the rate of development of the tensile strength of the forming clot are generated from the first derivative of the waveform. These parameters were first described by Sorensen et al.[28] and are provided by the TEG® system software (Figure 3). In order to correlate Total Thrombus Generation (TTG), that is the total area under the velocity curve, with thrombin generation (TG) using the TAT reference method, we measured the TEG® TTG parameter in various incremental segments of the developing TEG® waveform (Figure 4) and compared it to TAT measured for that same segment.

Specifically, a whole blood sample was run in the TEG® analyzer to obtain TEG® TTG, and the sample run was stopped at various time intervals ranging from 4 minutes to 40 minutes (Figure 4). When the TEG® run was stopped, the sample was immediately removed from the analyzer, and thrombin production was quenched within 10 seconds as described in Materials and Methods section. This is referred to as a TEG® segment and a TAT segment, respectively, and represents a single time point.

The first derivative of the TEG® segment determines the total thrombus generation, TTG, of that segment, while the corresponding TAT segment determines total thrombin generation,

TG. For the four volunteers – two male and two female – a total of 41 segments were generated, and their corresponding TTG and TAT values were computed. Linear and non-linear regressions were performed using the TAT variable as the dependant y-variable and the TTG variable as the independent x-variable to obtain the least-squares fit and the corresponding R^2 value between the TAT and TTG variables; test of significance was based on $\alpha \leq 0.05$.

RESULTS

As shown in Figure 5, fitting linear regression through the origin for the TTG variable vs. the TAT variable gives a correlation of R^2=0.7234 and prediction equation of y=6.3319x. Analyzing the same data with non-linear regression gives a correlation of R^2=0.8787 (Figure 6) between the TTG variable and the TAT variable at $P \leq 0.0001$ and prediction equation of $y=3122e^{0.0005x}$. Linearizing using the natural log of the TAT variable produces the same R^2 of 0.8787 and prediction equation of y=0.0005x+8.0465, both indicating a strong correlation with the TTG variable at P=0.0001 (Figure 7). Figure 7 shows the majority of the data points along the regression curve at a wide range of thrombin generation as measured by the TAT reference method (LnTAT). Figure 8 shows a typical example of a TAT generation curve superposed on the TEG® tracing of the same specimen.

DISCUSSION

The quantitative measurement of thrombin generation has recently become a highly valued tool to assess the risk of bleeding or of thrombosis[6]. The use of a test based on whole blood seems to be desirable, in order to mimic as much as possible the in vivo conditions.

Thromboelastography has been used extensively over the last five decades to assess the kinetics of blood coagulation in whole blood, but no data exist to correlate the waveform tracing of blood coagulation generated by this technology and the kinetics of generation of thrombin. Several approaches have been used to initiate coagulation in the TEG system. We have elected to initiate coagulation with diluted tissue factor in order to create in vitro conditions as comparable as possible to in vivo environment. To this end, we have also used corn trypsin inhibitor in order to minimize the contribution of the contact system for initiation of coagulation, as severe deficiency of proteins of the contact system do not seem to have a clinically relevant role in blood coagulation. Contrariwise to experience reported by others[28], in preliminary experiments (data not presented) we have seen a significant retardation of the clotting time with the concentration of corn trypsin inhibitor used in our system; this suggests that, in our working conditions, the contact pathway of initiation of coagulation is blocked by corn trypsin inhibitor, and thrombin generation is initiated by the factor VIIa-tissue factor pathway. Once thrombin is generated through the initiation of coagulation by the interaction of trace amounts of activated factor VII with tissue factor, platelets are activated and support further generation of higher concentrations of thrombin, which lead to the formation of fibrin[29–30]. The end result of this complex system is the formation of a fibrin clot. Therefore, it is not surprising that the velocity profile of the TEG® system, an instrument that allows acquisition of continuous quantitative information on the developing clot, correlates with thrombin generation. The thrombin generation curve as measured by the TAT reference method and the thrombus generation curve as generated by the TEG® system are interrelated. In this study, Figure 7 shows that the majority of the generated data points representing the two variables are within the prediction intervals of the regression line. In addition, the high magnitude of R^2 at $P \leq 0.0001$ and the high significance of the prediction equation demonstrate the high efficacy of the prediction of thrombin generation, as measured by the TAT method, by the TEG® TTG variable.

AS shown in Figure 8, the first evidence of formation of fibrin manifested by deflection of the TEG® tracing takes place at a time when a very small proportion of the total TAT generation has occurred. This is consistent with our previously reported observation that a clot is formed in whole blood when less than 5% of the total thrombin has been generated[31].

In conclusion, we believe that our data demonstrate a clear and physiologically relevant correlation between the velocity of thrombus tensile strength generation expressed by the TTG numerical values and the velocity of thrombin generation expressed by TAT numerical values. We believe that, for precise studies of the kinetics of the clotting process in whole blood, direct measurement of thrombin generation assessed by TAT complex is currently the best test available. For clinical practice where test have to be simple and results have to be available rapidly, TTG is a reasonable proxy for assessment of thrombin generation.

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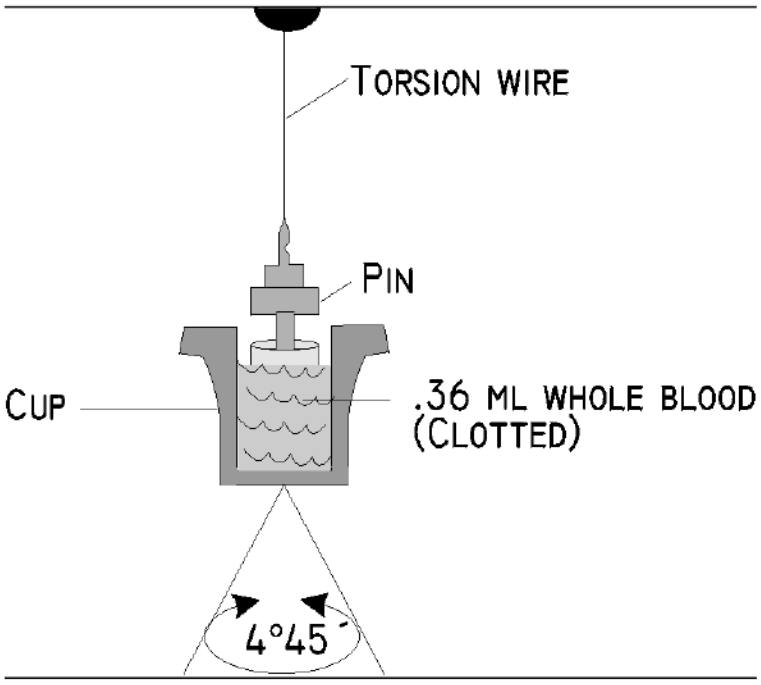

Figure 1.
The TEG® analyzer measures the clot's physical properties by the use of a special stationary cylindrical cup that holds a 360-μl sample of whole blood and is oscillated through an angle of 4° 45′. Each rotation cycle lasts ten seconds. A pin is suspended in the blood by a torsion wire and is monitored for motion. Thus, the magnitude of the output is directly related to the kinetics and the strength of the formed clot. As the clot retracts or lyses, these bonds are broken and the transfer of cup motion is diminished.

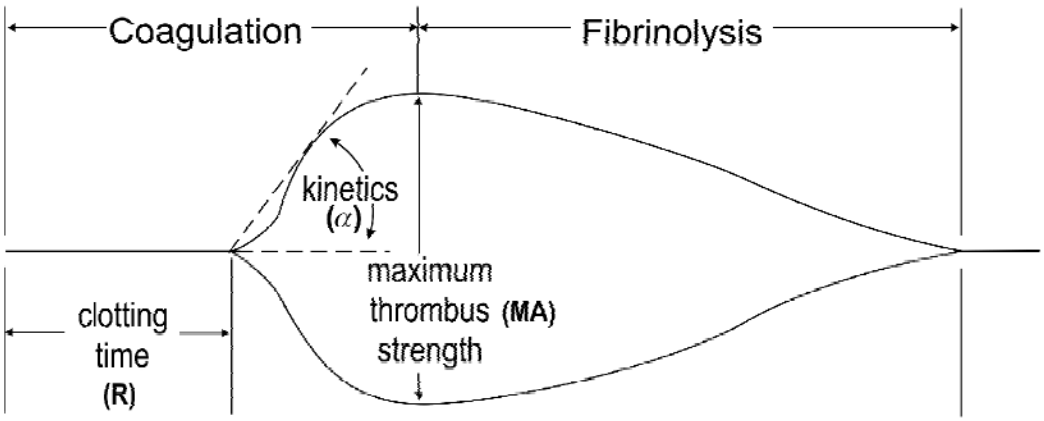

Figure 2.
Schematic representation of TEG® tracing with its principal parameters.

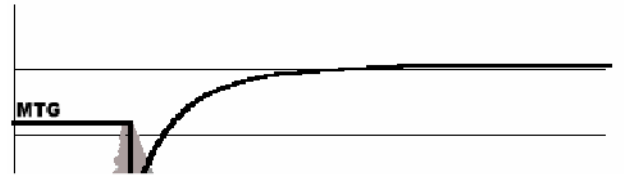

Figure 3.

A representative TEG® tracing obtained during the clotting process of whole blood of a normal subject. The shaded area represents the corresponding first derivative or velocity of the waveform produced by the TEG® system. Several parameters corresponding to the rate of development of the tensile strength of the forming clot are derived from the first derivative of the waveform.

The parameters are:

MTG – Maximum Rate of Thrombus Generation (100*mm/sec)

TTG – Total Area Under the Curve; Measures Total Thrombus Generation (mm)

TMG – Time to Maximum Rate of Thrombus Generation (sec)

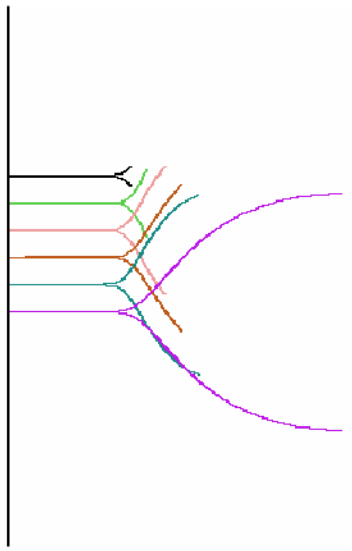

Figure 4.
TEG® segments representing sequential time points when the whole blood sample was stopped at various time intervals. The first derivative (velocity) of each segment determines the total thrombus generation, TTG, of that segment, while the corresponding TAT segment determines total TAT generation.

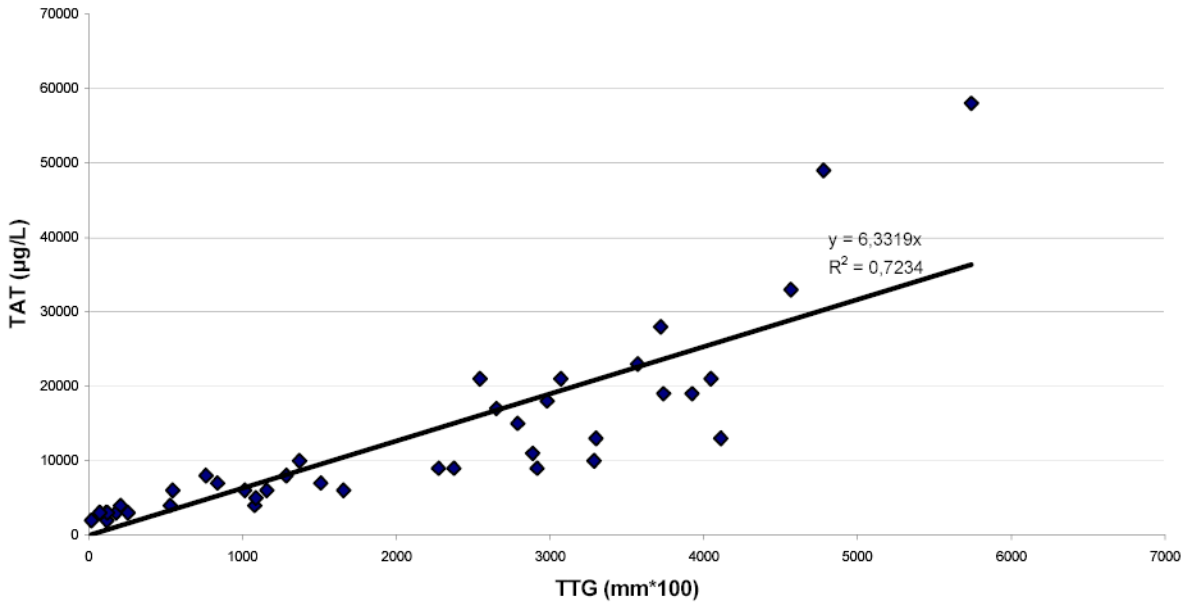

Figure 5.
Linear regression through the origin of Total Thrombus Generation, the TTG variable, versus TAT Generation, the TAT variable.

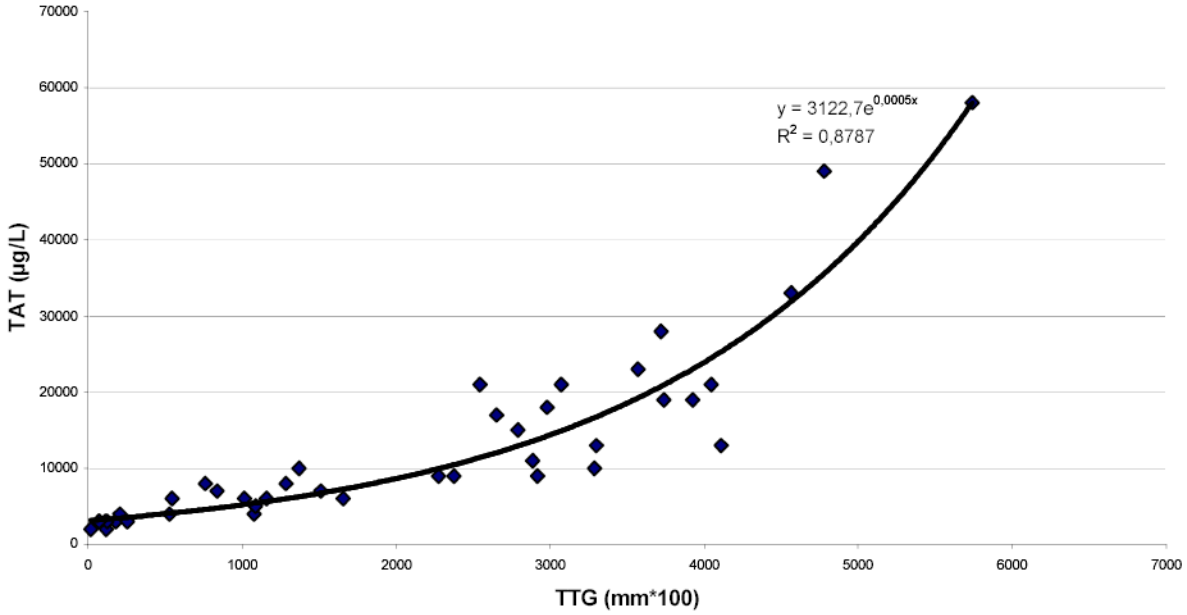

Figure 6.
Non-linear regression of Total Thrombus Generation, the TTG variable, versus TAT Generation, the TAT variable.

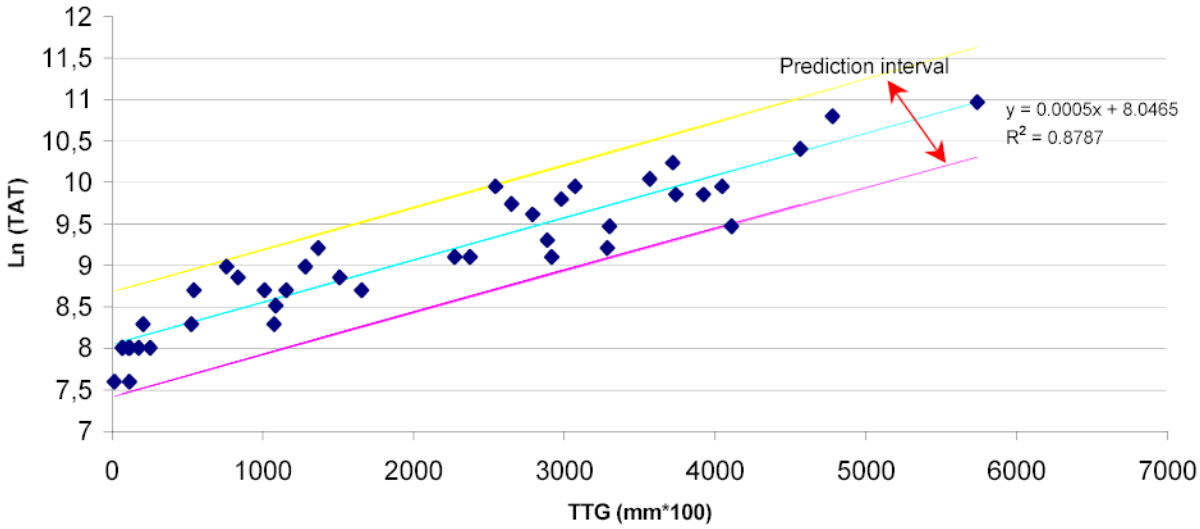

Figure 7.
Linear regression of Total Thrombus Generation, the TTG variable, versus the natural log of TAT Generation, the TAT variable. The majority of the data-points are within the prediction intervals of the regression line.

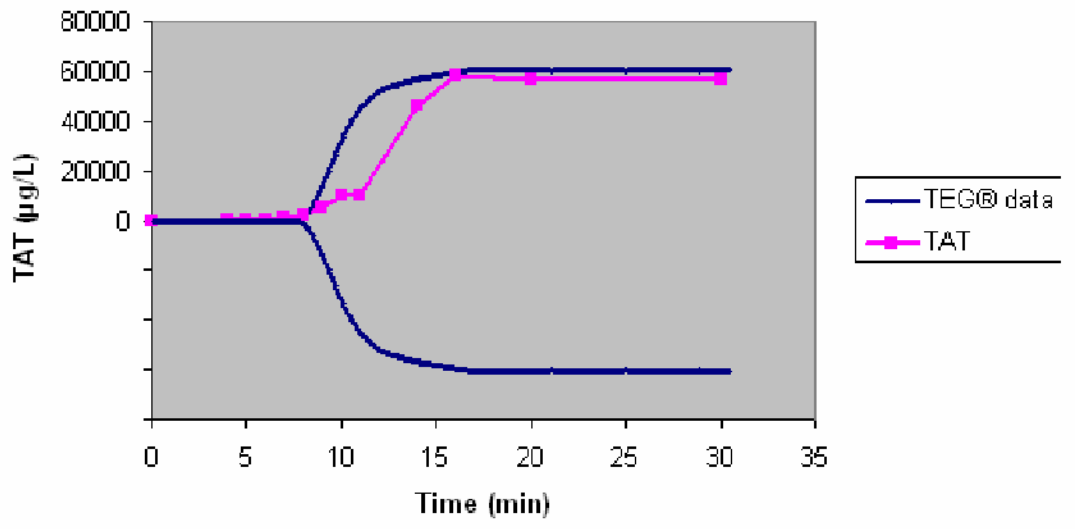

Figure 8.
Superposition of the TAT generation curve over the TEG® tracing of whole blood from a normal control.

Table 1

Formal Definition of TEG® Parameters

Clotting time	R	The period of time of latency from the time that the blood is placed in the TEG® analyzer until the initial fibrin formation. This represents the phase of initiation of the coagulation process.
Thrombus kinetic	α	A measure of the rapidity (kinetics) of fibrin build-up and cross-linking, that is, the rate of thrombus propagation.
Thrombus strength	MA	A direct function of the maximum dynamic properties of fibrin and platelet bonding via GPIIb/IIIa and represents the ultimate strength of the fibrin clot.
Thrombus lysis	LY	A measure the rate of thrombus breakdown that gives an indication of how the clot retracts and/or is lysed.