

# Delicate interactions between plasma factors and blood cells affect thrombin generation

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## Summary

This thesis focuses on the innovation of thrombin generation (TG) assays and the application of TG assays in clinical and basic hemostasis research. In **chapters 2-4** we described the development and validation of a novel whole blood (WB)-TG assay for studying the involvement of blood cells in coagulation, as well as the exploration of the WB-TG capacity of cirrhotic patient using an optimized near patient WB-TG prototype. Furthermore, **in chapter 5** we reviewed currently available evidence on the added value of blood cells in TG testing. In **chapters 6 & 7** we used plasma-TG assay for the characterization of coagulation phenotype in people living with HIV (PLHIV) and explored the use of TG as an intermediate phenotype to discover novel coagulation-related genetic variants, respectively.

In **chapter 2**, we developed a novel fluorogenic WB-TG assay with good reproducibility<sup>[1]</sup>. This assay is a major step forward compared to previous WB-TG assays that had a poor reproducibility<sup>[2]</sup>, or were prone to contact activation induced by the filter paper in the assay<sup>[3]</sup>. Platelet and erythrocyte counts, as well as platelet (in)activation were found to be crucial determinants of WB-TG, supporting our hypothesis that our WB-TG assay rightfully includes the influence of blood cells on coagulation. A strong enhancing effect of erythrocytes on the velocity of TG was observed, even in the presence of high platelet numbers. We also explored the performance of this assay in a healthy population and studied it in relation to age, gender, oral contraceptive use and blood cell count, which could serve as useful references for future studies.

In **chapter 3**, we studied WB-TG profiles of cirrhotic patients with an optimized near patient assay<sup>[4]</sup> and found that the TG capacity of these patients was comparable to that of normal people, suggesting a normal coagulability, in line with the widely accepted concept of rebalanced hemostasis in these patients<sup>[5]</sup>. Interestingly, the WB-TG velocity was slower in cirrhotic patients despite an intact WB-TG capacity. This observation might be explained by our previous results in reconstituted blood that counts of erythrocytes and platelets impact the TG velocity and capacity differently, thus a mild decrease of platelet and erythrocyte counts in cirrhosis only impaired the velocity of WB-TG but not the total capacity. The balance of the coagulation system in cirrhotic patients is very fragile and may easily tip towards a bleeding or a thrombosis phenotype. By using WB-TG a likely better representation of the in vivo situation is established, and WB-TG might be the assay of choice to study influences of therapy on fragile equilibria such as with liver cirrhosis and to predict their bleeding or thrombotic risk.

In **chapter 4**, We found an inhibiting role of erythrocytes on the anticoagulant function of activated protein C in WB-TG. The protein C system is an important anticoagulant pathway and impairment of this system is a common cause of thrombosis<sup>[6]</sup>. Higher erythrocyte count was related with a reduced anticoagulant effect of active protein C and thrombomodulin in WB-TG, both in a healthy population and in reconstituted blood samples. This effect was not dependent on platelets and was likely related with the phospholipid composition of erythrocytes. The inhibiting role of erythrocytes on APC function, combined with the observation of the enhancing effect of erythrocytes on WB-TG velocity, might provide a possible explanation for the increased thrombotic risk related with increased erythrocyte counts, for example polycythemia and erythrocyte infusion<sup>[7, 8]</sup>.

In **chapter 5**, we reviewed currently available continuous TG tests that can reflect the involvement of blood cells in coagulation, in particular the fluorogenic assays that allow continuous measurement in

platelet rich plasma and whole blood<sup>[9]</sup>. We also provide an overview about the influence of blood cells on blood coagulation, with emphasis on the direct influence of blood cells on TG. Platelets accelerate the initiation and velocity of TG by phosphatidylserine exposure, granule content release and surface receptor interaction with coagulation proteins. Erythrocytes are also major providers of phosphatidylserine and erythrocyte membranes trigger contact activation. Furthermore, leukocytes and cancer cells may be important players in cell-mediated coagulation because, under certain conditions, they express tissue factor, release procoagulant components and can induce platelet activation. We argue that testing TG in the presence of blood cells may be useful to distinguish blood cells-related coagulation disorders.

In **chapter 6**, we studied the plasmatic coagulability of HIV-infected individuals on combined antiretroviral therapy and found that the plasma TG capacity of these individuals was lower than healthy controls<sup>[10]</sup>. This observation, together with their reduced prothrombin levels and increased markers of inflammation and endothelial activation, suggest that the increased thrombotic risk of these individuals was not due to hypercoagulability and was most likely related to increased stimulation of coagulation by endothelial activation and inflammation during HIV infection. We also found that abacavir-use was associated with a prothrombotic TG profile compared to non-abacavir regimens, irrespective of age, sex and inflammation, thus providing new data for the debated thrombotic effect of abacavir <sup>[11]</sup>.

In **chapter 7**, we used TG as an intermediate coagulation phenotype in a genome wide association study and discovered that the *KLKB1* gene was related to the anticoagulant function of the protein C system. Functional experiments showed that in vitro supplementation of kallikrein augments the anticoagulant function of TM and APC in TG. This provides a possible mechanism for the previously observed association between the *KLKB1* gene and thrombosis <sup>[12, 13]</sup>. This also reinforces that the TM-modified TG assay could serve as a tool to discover novel mutations related to the protein C pathway.

In conclusion, in this thesis we presented innovative WB-TG assays that allow direct measurements of the influence of blood cells on TG. Although more studies on the standardization and clinical relevance of TG assays are needed, our preliminary results suggest that plasma- and WB-TG assays have promising applications in fundamental and clinical research in thrombosis and hemostasis.

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