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Review Article

Thrombin generation assay as a biomarker of cardiovascular outcomes and mortality: A narrative review

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ABSTRACT

Cardiovascular diseases (CVDs) are currently the leading cause of death worldwide. Therefore, there is interest in the search for cardiovascular risk markers that contribute to the early diagnosis, monitoring and prevention of cardiovascular events. Considering that CVDs present in their pathophysiology a strong interaction between inflammation and hemostasis, thrombin, a key enzyme in the clotting process can be thought as a possible biomarker of cardiovascular risk. The thrombin generation assay (TGA) by the Calibrated Automated Thrombogram (CAT) method has been used in numerous prospective studies. It is a relatively recent laboratory tool capable of globally evaluating the functioning of the hemostatic system through the determination of thrombin generation for investigating the contribution of procoagulants and natural anticoagulants, in addition to the effect of different drugs and a range of factors that interfere in this system. The analysis of thrombin generation can be a promising tool for estimating the risk of thrombotic diseases, although the association of TGA with arterial thrombosis has only recently attracted interest and remains to be better understood. The association between thrombin generation and cardiovascular events, especially acute myocardial infarction (AMI) and stroke, all-cause and cardiovascular mortality is still poorly investigated and the results are often inconsistent. Assessing the relationship between TGA and CVDs may not only contribute to increasing knowledge of the pathophysiological process that leads to coronary and cerebrovascular diseases, but may also suggest a new approach to prevention. In this article we review and summarize the results of the main studies that evaluated whether TGA parameters were associated with cardiovascular events, cardiovascular mortality and all-cause mortality. Possible contributing factors to the observed inconsistencies were also speculated.

1. Introduction

Chronic non-communicable diseases, of which cardiovascular diseases (CVDs) represent approximately 50 %, are the leading cause of death in the world at the moment [1]. Early diagnosis of CVDs is a public health strategy that can contribute to minimizing the social and economic impact of these diseases, reducing the incidence of severe and fatal cases, in addition to assisting in the monitoring of individuals at greater risk of developing such diseases [2]. In addition to assessing the lipid profile, there is a constant search for non-invasive cardiovascular risk markers that are accurate, effective, easy to perform and interpret allowing their clinical application, which would decisively contribute to early diagnosis, monitoring and prevention of cardiovascular events [3–6].

It is known that the hypercoagulable state caused by the imbalance between coagulation factors and natural anticoagulants, is a marker of cardiovascular risk [7]. The strong interaction between inflammation and hemostasis has placed thrombin as a possible biomarker of cardiovascular risk, which can be evaluated through plasma samples [4,6,8]. Atherosclerosis is a chronic inflammatory vascular disease in which there is a simultaneous participation of the coagulation and inflammation system and that may have as an outcome the formation of a thrombus that promotes vascular occlusion [6].

Thrombin is the key coagulation protein that under physiological conditions acts as a procoagulant and anticoagulant in order to prevent excessive clot formation. Thrombin activates pro-inflammatory

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signaling pathways through protease activated receptors (PARs) [PAR-1, PAR-3 and PAR-4], while thrombin-activated protein C (APC) activates anti-inflammatory and anti-apoptotic pathways through PAR-1. In atherosclerosis, the hemostatic balance is disrupted. APC is decreased by NF-kappa signaling and pro-inflammatory functions of thrombin prevail. The result is the activation of different proteins [monocyte chemotactic protein-1 (MCP-1), E/P-selectin, vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecules-1 (ICAM-1) and cells [endothelial cells, vascular smooth muscle cells, smooth muscle cells, platelets] involved in the atherosclerosis [7].

There are still no routine laboratory tests that are good evaluators of the hypercoagulable state. The tests most commonly used to assess hemostasis are prothrombin time (PT) and activated partial thromboplastin time (APTT), which are useful in diagnosing bleeding disorders and monitoring the use of anticoagulants (warfarin and heparin, respectively). In contrast, the thrombin generation assay (TGA) is a diagnostic method used in research for about the last 20 years is able to assess hemostasis broadly covering the stages of initiation, amplification and propagation of coagulation and taking into account the pro- and anticoagulant forces of the hemostatic system and is able to detect the hypercoagulable status [9–11], however lacks routine application because of issues like standardization, automation and indication for clinical implementation [12]. Studies performed with different populations have reported that thrombin generation is associated with incidence of cardiovascular events [13–15], all-cause mortality [14,16] and/or mortality from cardiovascular causes [17]. However, the findings in the literature are still inconsistent regarding the existence of the aforementioned association, as well as its direction.

Therefore, in this brief article we reviewed and summarized the results of studies that evaluated whether TGA parameters were associated with cardiovascular events, cardiovascular mortality, and all-cause mortality. In addition, we discussed the current status of TGA in clinical trials and attempted to outline the perspectives for this methodology in clinical use.

2. Search strategy

The article search was performed on PubMed, Science Direct and Embase, between January and June 2022. We used the terms “cardiovascular disease,” “cardiovascular mortality,” “all-cause mortality”, “stroke”, “myocardial infarction” and “thrombin generation” in several settings. Only articles in English were included. In addition, relevant articles with complementary information cited in the selected articles were also included.

3. Thrombin generation assay (TGA) using Calibrated Automated Thrombogram (CAT) method

To assess thrombin generation in vivo, some coagulation assays can be used, such as the measurement of plasma levels of prothrombin fragments 1 + 2 (F1 + 2), thrombin-antithrombin complex (TAT) e D-Dimer. When the plasma levels evaluated by these assays are high, a state of hypercoagulability is present, that is, a pathological activation of coagulation in vivo [6,18]. Another possibility to measure thrombin is through in vitro thrombin generation assays that assess the potential for thrombin production in plasma after induction by a triggering agent [7]. Thus, in vivo thrombin generation assays measure the amount of thrombin formed in the body reflecting a possible ongoing pathology [19]. In vitro thrombin generation assays assess the ability of a plasma sample to generate thrombin after activation of coagulation, whose generation is counterbalanced by the natural anticoagulation mechanism, drugs or other factors that influence the hemostatic system. Thus, in vitro assays are useful to obtain information on the complex balance between pro and anticoagulant forces and to identify possible hemostatic disorders [20,21]. Thus, TGA is more representative of the physiological state and may better reflect the hemostatic phenotype, whether hypo or hypercoagulable [9,11].

Developed by Macfarlane and Biggs in the 1950s and modified by Hemker in the 1980s, the TGA has shown to be an important tool for the assessment of thrombotic and hemorrhagic risk, being used and evaluated in numerous clinical conditions in the last 15–20 years [10,22]. In addition to its use in clinical research, as reviewed by Depasse et al. [23], TGA has also been a very useful tool in basic science contributing to increase our understanding of the dynamics of blood clotting beyond conventional laboratory methods. Also, according to the same authors, TGA has also been applied in toxicological studies, drug development and elucidation of the mode of action of drug substances, among other applications. Since TGA is an open system, it has enormous potential for studies related to hemostasis, such as the development of new procoagulant or anticoagulant drugs. Our group has used TGA to investigate the action of snake venoms [24] and plant extracts [25] from the rich Brazilian biodiversity on the hemostatic system.

The CAT method, in its semi-automated and automated versions, has been the most used method to assess thrombin generation [12,26]. It is a method that continuously measures the proteolytic activity of thrombin formed in plasma for 60 minutes using a fluorogenic substrate. A solution of tissue factor (TF) + phospholipids and a solution containing fluorogenic substrate + calcium are added to the plasma samples, initiating the activation of coagulation and subsequent formation of thrombin. Thrombin cleaves the fluorogenic substrate, releasing a fluorophore that has its fluorescence intensity measured by a fluorimeter [10]. The intensity of fluorescence is proportional to the amount of thrombin produced. Continuous comparison with a known concentration calibrator allows converting the rate of change of the fluorescent signal into a thrombogram, which is a curve of thrombin concentration over time. The thrombogram is characterized by presenting a latency phase (lagtime), in which a small amount of thrombin is produced, followed by an explosive rise to the peak (propagation) and by an equally rapid decline in its production (thrombin inhibition by the of natural anticoagulants) [27–29].

Continuous comparison with the calibrator reduces the effect of light absorption from other molecules (“internal filter effect”), corrects for plasma colour, substrate depletion and fluorescent signal quantification [19].

The main parameters of TGA are lagtime, time-to-peak, peak and endogenous thrombin potential (ETP). Lagtime and time-to-peak are parameters that indicate the time between the addition of the triggering agent and the beginning of thrombin production and the time until maximum production (peak) is reached, respectively. Peak and ETP are parameters related to the amount of thrombin produced during the reaction, with peak being the maximum concentration produced, while ETP is the area under the curve and refers to the total amount of thrombin generated over the period of 60 minutes, representing the balance between procoagulant and anticoagulant forces [29,30]. Other parameters less mentioned in the articles are time-to-tail, which indicates the time from the beginning of the reaction to the tail [14] and the velocity index (velocity index-VI) which is defined by the equation [peak/ (time-to-peak – lagtime)] and represents a composite index that includes the latency phase and the time-to-peak [11]. In general, hypercoagulable states are characterized by lower lagtime and time-to-peak and higher peak and ETP, while hypocoagulable states are generally related to prolonged lagtime and time-to-peak and lower peak and ETP [29,30].

In order to reduce the variability between assays and laboratories, Dargaud and collaborators published a series of articles for the elaboration of a standardized protocol for performing the TGA by the CAT method. Within this protocol, the normalization of the results obtained against a reference sample was proposed. Normalization consists of the ratio of the parameter measured in the sample against the same parameter measured in a pool of normal samples or a commercial pool [31–33]. ETP and peak are the most frequently normalized parameters.

In 2021, after research conducted by the International Society on

Thrombosis and Haemostasis (ISTH), Ninivaggi et al. [12] developed a guide for measuring thrombin generation in clinical conditions. The researchers' goal is to reduce inter-laboratory variability and facilitate the use of TGA in patient management. This guide contains recommendations on blood collection; sample handling, processing and storage; concentration and sources of reagents; sample dilution conditions and temperature; test calibration and replication; calculation and interpretation of results, in addition to reference values.

The TGA can be performed under different conditions for evaluation of the hemostatic system depending on the purpose of the study, constituting a very versatile test [23]. It is possible to use platelet-poor plasma (PPP), platelet-rich plasma (PRP) or whole blood as a biological sample for the assay [34]. Through PPP, TGA allows detecting deficiencies of all clotting factors (except factor XIII), the action of oral anticoagulants (warfarin, and factors Xa and IIa inhibitors – DOAC's) and heparin, among other applications. Furthermore, in PPP, it is possible to evaluate the function of natural anticoagulants, i.e., antithrombin, protein S and protein C. For example, the addition of thrombomodulin to the plasma sample allows investigating the function of activated protein C. Another example is the evaluation of thrombin generation in PPP of hemophiliac patients undergoing therapy with emicizumab, through the use of contact pathway activator containing triggering reagents, more specifically FXIa or ellagic acid [35].

On the other hand, in PRP, it is possible to assess the combined activity of plasma coagulation factors and platelets. Therefore, the function of membrane receptors presents on platelets, as well as the effect of antiplatelet drugs can be evaluated by TGA in PRP [29,30]. As for the use of whole blood for TGA, Wan et al. [36] have extensively discussed the involvement of blood cells in clotting, that is, platelets, erythrocytes and leukocytes, which would make the test more sensitive to hemostatic changes. According to these authors, platelets accelerate the initiation and rate of thrombin generation due to exposure of phosphatidylserine on their surface, release of granule content and interaction of surface receptors with coagulation proteins. In addition, erythrocytes also provide phosphatidylserine and leukocytes, under certain conditions, express tissue factor, release procoagulant components and can induce platelet activation. TGA in the presence of blood cells may be useful in distinguishing blood cell-related clotting disorders [36].

Another modifiable factor in TGA is the concentration of TF + phospholipids used, whose variation aims to study specific clinical purposes [23]. According to these authors, at low concentrations of TF (0.4 to 2 pM) + phospholipids (0.4 to 4 μM), TGA better evaluates the intrinsic pathway and bleeding tendency; while at higher concentrations of TF (between 5 and 40 pM), depending on the concentration of phospholipids used, TGA better assesses thrombophilic states; and, finally, to assess the use of anticoagulation, high concentrations of TF (between 20 and 50 pM) + phospholipids (~4 μM) are recommended [28]. It is known that lower amounts of TF increase the sensitivity of the CAT method, however, it is more influenced by the activation of the contact pathway, which can occur during blood collection, and would influence the results [31].

Due to the sensitivity of TGA to conditions of hypo and hypercoagulability, this method has a wide range of applications in research and in the clinic, both for diagnosis, assessment and monitoring of bleeding disorders and for venous and arterial thrombotic risk assessment [30]. Assessing the relationship between TGA and CVDs may not only contribute to improving knowledge of the pathophysiological process that leads to coronary and cerebrovascular diseases, but may also suggest a new approach to prevention [13].

4. TGA, cardiovascular events and mortality

The identification of survival prognostic markers in vulnerable populations, elderly or with chronic conditions is important for health care planning [4].

The analysis of thrombin generation is a promising tool for estimating the risk of thrombotic diseases, but the association of TGA with arterial thrombosis has aroused recent interest and still needs to be better understood, since contradictory results have been found [13–17,37–43].

In a cross-sectional study with 295 individuals undergoing angiography for suspected coronary artery disease (CAD), Borissoff et al. [38] found no differences regarding ETP values between participants with and without CAD. However, they observed a U-shaped association between ETP and stratification of CAD, i.e., mild CAD was associated with lower levels of ETP and severe CAD was associated with high levels of ETP, indicating that early in atherosclerotic lesion there is an increase in thrombin generation compared to advanced stable lesions.

Loeffen et al. [39] performed a prospective cohort study with 104 patients with suspected acute coronary syndrome (ACS) and 42 individuals without ACS (for whom no criteria for diagnosis of ACS were found), transported by ambulance to the Medical Center of the Maastricht University or to the Atrium Medical Center at Heerlen Hospital, Netherlands. Of the 104 participants, 73 individuals with early-stage ACS (AMI with or without ST-segment elevation or unstable angina) were followed for 6 months. The TGA was performed before the administration of any medication, and subsequently 1 and 6 months after the inclusion, and the association with the occurrence of cardiovascular death, recurrence of AMI or a second coronary intervention (percutaneous coronary intervention or coronary artery bypass graft surgery) and stroke between one and six months after entering the study was evaluated. It was observed that, in the acute phase, individuals with ACS had a prothrombotic profile compared to individuals without ACS, as significantly lower values of time-to-peak and time-to-tail and higher values of peak and VI were found. A significant reduction between ETP values measured 6 months after the ACS in comparison to the acute phase. Higher baseline peak values were significantly associated with a higher risk of recurrence of cardiovascular events. The other TGA parameters were not significantly associated with the recurrence of cardiovascular events.

In a sub-study of a prospective cohort study in the Maastricht University Medical Center, Breet et al. [44] investigate whether thrombin generation provides additional insight into the assessment of bleeding risk for high clinical-risk patients using dual antiplatelet therapy [combination of low-dose aspirin (80–100 mg) and a P2Y12 inhibitor (clopidogrel 75 mg, prasugrel 5–10 mg, or ticagrelor 90 mg)] for a planned duration for more than months. TGA were measured in 93 high clinical-risk frail patients using dual antiplatelet therapy after percutaneous coronary intervention (PCI) followed for a 12-month period. Thrombin generation at 1 and 6 months after PCI was compared between patients with and without bleeding events. One month after PCI, thrombin generation was significantly lower in patients with bleeding in the following months compared to patients without bleeding (lower ETP, peak and VI and higher time-to-peak and lagtime). At 6 months follow-up, peak height and VI were still significantly decreased in the bleeding group as compared to non-bleeders. They concluded that high clinical-risk patients using dual antiplatelet therapy with clinically relevant bleeding during follow-up show reduced and delayed thrombin generation possibly due to variation in coagulation factors. Thus, impaired thrombin generation potential may be associated with dual antiplatelet therapy, increasing the bleeding risk in high clinical-risk patients.

Smid et al. [37], in the GLAMIS study, evaluated the generation of thrombin in 171 individuals with AMI between three and nine months after the cardiovascular event, minimizing acute phase influences, matched by sex and age with 185 healthy controls. The authors observed that the lagtime, normalized ETP (nETP) and normalized peak (npeak) were significantly higher in participants who had AMI compared to controls. They also found a weak but significant positive correlation between lagtime, nETP and npeak and cardiovascular risk factors, such as body mass index (BMI), total cholesterol and triglycerides. Finally, they concluded that the increase in the values of nETP and npeak after

AMI, is an important characteristic and indicative of hypercoagulable state in patients with coronary heart disease suggesting a risk of a new arterial thrombotic event.

Thrombin generation was also evaluated in the PROSPER Study. This is a double-blind, randomized, placebo-controlled study that evaluated the use of pravastatin 40 mg/day to reduce cardiovascular events in 4932 elderly people aged 70 to 82 years with preexisting CVDs or with risk factors such as smoking, diabetes or hypertension. The association between TGA parameters and the risk of CAD and stroke was evaluated. Loeffen et al. [14] found an inverse association between nETP and npeak at baseline and the occurrence of stroke after 3.2 years of follow-up. The lagtime and time-to-peak parameters were directly associated with the occurrence of stroke. To determine the influence of treatment (pravastatin or placebo) on thrombin generation measurements, interaction tests were performed, but no significant interaction was demonstrated with any of the TGA parameters. The authors justified that the lower ex vivo thrombin generation observed may have been preceded by an increase in in vivo thrombin generation, which would have consumed the clotting factors available in the plasma. No significant association was found between TGA and CAD. Regarding mortality, they observed an association between general and cardiovascular mortality and the highest values of the lagtime and peak. However, after adjustments for inflammatory markers (interleukin-6 and C-reactive protein) this association lost significance, reaffirming the relationship between hemostasis and inflammation.

Carcaillon et al. [13] in a case-cohort study developed within The Three-City study, evaluated the association of thrombin generation with CAD and acute ischemic stroke in elderly people aged 65 years or older without previous cardiovascular events. TGA was performed in 186 participants who developed CAD (angina, coronary dilation or bypass, AMI and death from CAD), 87 participants who had stroke (ischemic, fatal or non-fatal), and 1177 randomly selected controls. After four years of follow-up, no significant associations were found between both ETP and peak, and CAD; however, higher ETP and peak values were associated with increased risk of stroke. These results were more relevant among women. The authors have suggested that TGA is an independent predictor for stroke especially among women, and that hypercoagulability may play an important role in the pathogenesis of this disease.

Rooth et al. [40] in a cohort of 205 individuals with stroke evaluated the generation of thrombin at hospital admission and after 30 days. They observed higher peak values at both times in participants with stroke compared to the healthy group. Regarding ETP, a significant difference was observed between participants with stroke and the control group only in the acute phase. ETP decreased significantly between the acute phase and day 30. The authors suggest that thrombin generation can be used to assess the risk of hypercoagulability in patients with stroke.

In cohort study with 190 individuals within 45–65 years with arterial ischemic stroke (AIS) or transient ischemic attack, Lundstrom et al. [41] evaluated if the generation of thrombin measured in the acute and the convalescent phases, would be associated with an increased risk of recurrent ischemic event fatal and nonfatal. ETP and peak in the acute phase were significantly lower for patients with primary outcome (fatal and nonfatal AIS or AMI) than for patients without this outcome. They found that high levels of ETP and peak in the acute phase were associated with a reduced risk of recurrent ischemic events. They also evaluated the prothrombin fragment F1 + 2, as a reflect of thrombin generation in vivo, and found that patients have reduced levels, suggesting that consumption of coagulation factors was not increased after an arterial ischemic event.

Donkel et al. [42], in the Genetic risk factors for Arterial Thrombosis at a young age: the role of TAFI and other Coagulation factors (ATTAC)' study, evaluated the generation of thrombin in 160 patients aged 18–55 years with a first acute arterial ischemic event (ischemic stroke or transient ischemic attack), and 160 healthy controls. To avoid a possible effect of acute phase response on coagulations parameters the thrombin generation was assessed between one and three months after the event. The authors observed that the lagtime, time-to-peak and time-to-tail were significantly lower in participants who had the ischemic event compared to controls. They also found that peak was higher while ETP was not different in these individuals compared to controls. Analyzing the thrombin generation curves, they observed a left deviation in patients, indicating that a thrombin production starts earlier, reaches the peak earlier and also terminates earlier. Finally, they found a significant association between lagtime, time-to-peak and time-to-tail and the ischemic event after the adjust for age, sex and cardiovascular risk factors. They concluded that the hypercoagulate state in young patients with ischemic event is relevant and a possible explanation for differences between young and elderly stroke patients could be due to the low prevalence of atherosclerosis in younger patients. In older patients, the atherosclerosis activates the coagulation system, producing thrombin, that stimulates the development of other atherosclerotic plates and more thrombin generation is produced in a cycle process.

In order to assess whether thrombin generation could predict the risk of cardiovascular events (death, recurrence of AMI, heart failure, and ischemic or hemorrhagic stroke) or bleeding events leading to hospital admission, Christersson et al. [15] evaluated 421 patients with AMI after hospital discharge. These patients were participants in the REBUS cohort Study and were followed for two years. In the univariate analysis, higher peak and ETP value were associated with a lower risk of stroke, however, after adjusting for confounding factors, none of them maintained this significant association. No significant associations were found between ETP and peak values and the other outcomes analyzed. Patients were treated according to international and national guidelines, at the discretion of the responsible physicians.

The absence of association between TGA and ischemic stroke was also found by Balogun et al. [43] in a cohort study that evaluated 170 participants admitted with suspected stroke and 71 healthy controls. The authors observed that lagtime and time-to-peak were prolonged in participants with ischemic stroke, however ETP and peak were not significantly different in relation to controls, not being useful for evaluating hypercoagulability in patients with stroke.

The association between TGA and cardiovascular or all-cause mortality was evaluated in Florence Acute Myocardial Infarction-2 (AMI-Florence2 Study) [17] and Gutenberg Health Study (GHS) [16]. In the AMI-Florence-2 cohort study involving 292 patients with ACS who underwent coronary angioplasty with stent implantation, Attanasio et al. [17] evaluated the association between cardiovascular death and TGA parameters, and they have observed that ETP, peak and velocity index were significantly higher after angioplasty in patients who died after two years of follow-up. These authors concluded that TGA parameters may be useful biomarkers for risk stratification after this surgical procedure.

Van Paridon et al. [16] evaluated the relationship between TGA parameters and cardiovascular risk factors, as well as the association with overall mortality in the GHS cohort study involving 4843 individuals aged 35 to 74 years. The authors found a significant and positive association between lagtime, ETP and peak, and obesity and dyslipidemia. Lagtime (with 1pM and 5pM of TF concentrations) and ETP (with 5pM of TF concentration) above the reference values proposed by the authors, were associated with worse survival. In Cox regression analysis, after adjustments for age, sex, vitamin K antagonist use, diabetes, obesity, smoking, hypertension, and CVDs history, lagtime (1pM TF) above the 95th percentile, and ETP (5pM TF) above the 95th percentile were predictors of mortality.

In view of the above content, it is clear that the relationship between thrombin generation and the occurrence of incident or recurrent cardiovascular events, fatal or not, is variable and, in some cases, contradictory. In the acute phase, right after a cardiovascular event, a prothrombotic profile is observed based on the TGA, given the increased levels of both ETP and peak. In the chronic phase "stable CVDs" there seems to be a hemostatic stabilization, since there is a reduction in the potential for thrombin generation, as shown at Fig. 1 below:

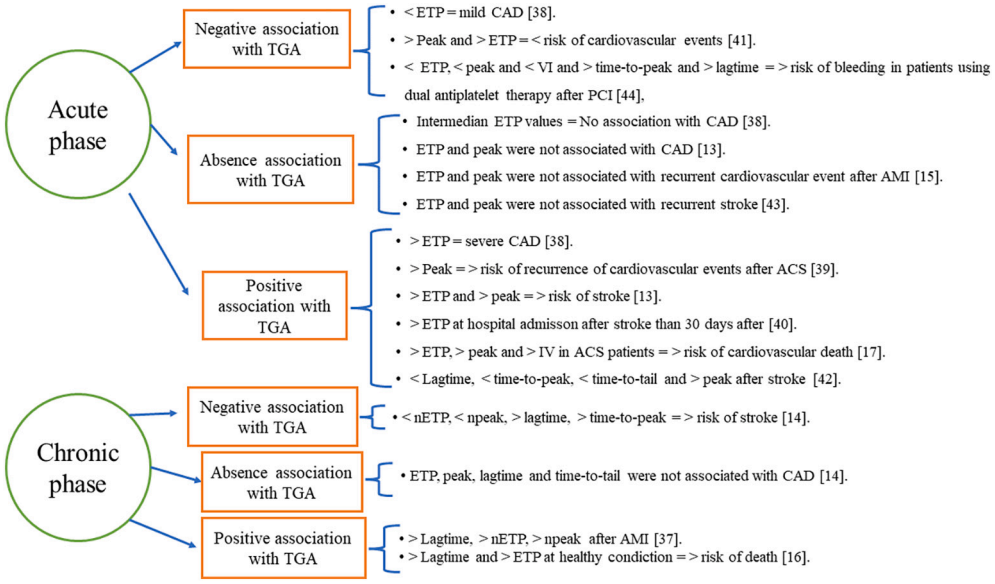

Fig. 1. Associations between TGA parameters during acute and chronic phases of CVDs.

The potential of TGA parameters as predictors of cardiovascular events and death remains to be established. Table 1 presents a summary of the main studies that evaluated the association between TGA performed by the CAT method, cardiovascular events and mortality. All studies cited in Table 1 used PPP as a sample.

5. Challenges and perspectives

Although studies show the role of thrombin in atherosclerosis and in the clinical manifestations of atherothrombotic disease [45–48] the clinical relevance of these associations and the causal effects found experimentally remain unclear and the studies are not always able to detect a hypercoagulable state using TGA [49].

Factors related to the collection, obtaining and type of plasma samples used, the development of the technique (different concentrations of TF and phospholipids, for example), the design and population of the study, and also the analyzed outcomes can make difficult to compare the results found. Sample collection and processing, in-house production or the use of commercial reagents and the variability of reagent concentrations are factors that can impact the evaluation of studies. The lack of standardization, especially in the pre-analytical stage, may interfere with the results of the several studies. Some authors did not inform the centrifugation conditions of the samples [15,38], others performed one centrifugation between 2000 and 3000 *g*, but the time and temperature of the procedure were more variable [13,16,37,41]. Attanasio et al. [17] and Breet et al. [44]centrifuged the samples twice at 2000 *g* for 5 minutes at the first study and for 10 minutes at the second, at room temperature, Donkel et al. [42] centrifuged the samples twice (the first at 2000 g for 10 minutes at 4 °C and the second at 20,000 g for 10 minutes at 4 °C), while Loeffen et al. [39] and Balogun et al. [43] performed three centrifugations, but under different conditions.

Regarding the population studied, most studies evaluated hemostatic markers in individuals with a history of CVDs. Only Carcaillon et al. [13] and Van Paridon et al. [16] worked with healthy populations, but in different age groups. The thrombin generation measured directly after a thrombotic event can differ significantly from thrombin generation measured months after the event, especially when anticoagulant treatment is started in between. Ciment et al. [50]) declare that the CAT method for thrombin generation potential analysis is sensitive to heparins and oral anticoagulant treatment even after cessation, which inhibited thrombin formation. Because of it the use of oral anticoagulation or low-molecular-weight heparins is a common exclusion criterion at studies that use CAT. all studies included in our review excluded participants using anticoagulation or the TGA was performed with samples collected before the administration of any medication.

Dual antiplatelet therapy is given to patients after an arterial thrombotic event. The vast majority of patients in the studies received antiplatelet treatment [15,17,37,39–41,43], but samples were collected prior to medication administration. Thus, the treatment may interfere with the outcomes found, but it did not interfere with the measurement of TGA parameters. Only Bree et al. [44] included participants using dual antiplatelet therapy, but the objective of this study was investigated whether thrombin generation measurement in plasma provides additional insight into the assessment of bleeding risk for high clinical-risk patients using dual antiplatelet therapy.

The heterogeneity of the study populations is undoubtedly a very important factor in the lack of consistency between TGA results and greater occurrence, severity and death from CAD, stroke and even recurrence of atherothrombotic events. In this context, in our view, different stages of the diseases that present hypercoagulability, occurrence of atherothrombosis in different vascular beds, other associated comorbidities and use of different medications, in addition to the genetic burden interfering with the hemostatic profile, are some of the factors that also contribute to the diversity of data reported in the literature.

Another important point is that the degree of hyperlipidemia can interfere with the potential for thrombin generation, leading to contradictory results. For example, Olivieri et al. [51] observed that only apolipoprotein C-III (Apo C-III), and not total cholesterol, low-density lipoprotein, triglycerides and Apo E was associated with ETP and peak and the Apo C-III was considered an independent risk factor for CAD. Thus, Ten Cate & Hemker et al. [6] suggest that these findings show that Apo C-III could have a functional role in thrombin generation.

It is important to highlight that thrombin has protective functions (generating APC), but also may elicit prothrombotic and proatherogenic functions, and the relationship between these functions depends on several factors, such as the balance between the binding of APC and thrombin to the PAR receptor, amount of meizothrombin (thrombin binds to phospholipid surfaces), the location of thrombin formation, among others [6].

In addition, it is known that platelets play an important role in the pathophysiology of CVDs and most studies use PPP as a sample. The physiological interactions between platelets and coagulation factors during the amplification phase of coagulation may be relevant in the assessment of the potential to generate thrombin, since thrombin

Table 1
Association between Thrombin Generation assays (TGA), and occurrence of cardiovascular events and mortality in the main studies.

Author/study	Type of study	Participants (n)	Population	Response variable/ outcome	TGA parameters	TGA Method	Number of evaluations	Centrifugation	Study findings/ association
Carcailon et al. [13]/Three-city study	Case cohort	1450	Elderly ≥65 years	Fatal and non-fatal CAD and stroke	ETP and peak	CAT (5pM TF)	1 time on baseline	1 centrifugation at 3.000 *g* at 4 °C (time not informed)	There was no significant association of both ETP and peak with CAD. Higher ETP and peak values were associated with increased risk of stroke. Stratified analysis by sex showed a more relevant association of both ETP and peak, and CVA in women.
Borissoff et al. [38]	Cross-sectional	295	Individuals undergoing angiography for suspected CAD	Correlation with presence and severity of atherosclerosis	ETP	CAT (5pM TF)	1 time	Uninformed	ETP values did not differ in the groups with and without CAD. U-shaped relationship was observed between ETP and the extent of CAD
Smid et al. [37]/ GLAMIS	Case control	356	AMI patients and healthy controls	Hypercoagulable state	Lagtime, nETP and nPeak	CAT (1 and 2pM TF)	1 time (between 3 and 9 months after AMI)	1 centrifugation at 2.000 g for 10 min at room temperature	Thrombin generation is altered in patients with AMI. A prolongation of lagtime and an increase in both nETP and nPeak were observed in participants who had AMI compared to controls.
Rooth et al. [40]	Cohort	258	Patients with stroke or ischemic attack within 2 weeks and healthy subjects	Hypercoagulable state	Lagtime, time-to-peak, peak and ETP	CAT (5pM TF)	2 times (on hospital admission and after 1 month)	1 centrifugation at 2.000 *g* for 20 min	Higher peak values were observed at both moments in patients with stroke. ETP values were significantly higher on day 0 compared to day 30 and controls.
Loeffen et al. [14]/ PROSPER Study	Cohort	4932	Elderly aged between 70 and 82 years with preexisting CVDs or with cardiovascular risk factor(s)	Occurrence of stroke or CHD and general and CVDs mortality	ETP, peak, lagtime and time-to-tail	CAT (1 and 5pM TF)	1 time on baseline	Uninformed	Lower nETP and npeak were associated with higher risk of stroke. Higher lagtime and time-to-peak were associated with higher risk of stroke. There was no significant association of TGA parameters with CHD and mortality.
Loeffen et al. [39]]	Cohort	146	104 patients with ACS and 42 subjects without ACS	Cardiovascular death, recurrence of AMI or a second coronary intervention or stroke.	ETP, peak, time-to-peak and VI	CAT (0 and 1pM TF)	1 time at baseline, and 1 and 6 months after the cardiovascular event	2 centrifugations at 2.000 *g* for 5 min followed by 1 centrifugation at 11.000 *g* for 10 min	Individuals with ACS had a shorter time-to-peak and higher VI and peak compared to controls. Higher peak was associated with higher risk of recurrence of cardiovascular events.
Attanasio et al. [17]/AMI-Florence2 Study	Cohort	292	Patients with ACS undergoing coronary angioplasty with stent implantation	Death by CVDs	ETP, peak and VI	CAT (5pM TF)	1 time on baseline	2 centrifugations at 2.000 g for 5 min at room temperature	Higher ETP, peak and VI values were associated with death.
Balogun et al. [43]	Cohort	241	170 patients after acute ischaemic stroke and 71 healthy volunteers	Hypercoagulable state	Lagtime, time-to-peak, peak and ETP	CAT (5pM TF)	1 time	2 centrifugations at 4.750 g for 10 min at room temperature and 1 centrifugation before performing the TGA	Lagtime and time-to-peak were prolonged in ischemic stroke. ETP and peak were not significantly different between stroke types or controls
Christersson et al. [15]/ REBUS Study	Cohort	421	Individuals with AMI after hospital discharge	Death, recurrence of AMI, stroke and heart failure, and bleeding.	ETP and peak	CAT (0pM TF)	At baseline (3–5 weeks after AMI) and 2–3 weeks after initiation of follow-up	Uninformed	Higher ETP and peak values were associated with lower risk of stroke, although this association lost

(*continued on next page*)

Table 1 (*continued*)

Author/study	Type of study	Participants (n)	Population	Response variable/ outcome	TGA parameters	TGA Method	Number of evaluations	Centrifugation	Study findings/ association
									significance after adjustments.
van Paridon et al. [16]/ GHS	Cohort	4843	Adults aged 35 to 74 years	General mortality	Lagtime, peak and ETP	CAT (1 and 5pM TF)	1 time on baseline	1 centrifugation at 2.000 *g* for 10 min at room temperature	Higher lagtime and ETP values were associated with mortality.
Lundstrom et al. [41]/ PROPPSTPPP study	Cohort	243	190 patients with stroke ischemic or transiente ischemic attack and 53 heathy controls	Fatal and nonfatal acute ischemic stroke or AMI	ETP and peak	CAT (5pM TF)	1 time on baseline	1 centrifugation at 2.000 *g* for 20 min at room temperature	Higher ETP and peak values were associated with reduced risk of recurrence os ischemic events.
Donkel et al. [42]/ATTAC' study	Case control	360	160 patients with stroke ischemic or transiente ischemic attack and 160 heathy controls	Hypercoagulable state	Lagtime, ETP, peak, time-to-peak, IV, time-to-tail	CAT (1pM TF)	1 time	1 centrifugation at 2.000 g for 10 min at 4 °C and 1 centrifugation for 20.000 *g* for 10 min at 4 °C	Lagtime, time-to-peak and time-to-tail were lower in participants who had the ischemic event. Peak was higher while ETP was not different in these individuals compared to controls
Breet el at. [44]	Cohort	93	15 patients with clinically relevant bleeding and 78 patients without bleeding	Relevant bleeding	ETP, peak, IV, lagtime, time-to-peak	CAT (1pM TF)	1 month after PCI and 6 months after PCI	2 centrifugations at 2.000 g for 10 min	Lower ETP, peak and VI and higher time-to-peak and lagtime one month after PCI in patients with bleeding compared to patients without bleeding. At 6 months follow-up, peak and VI were still significantly decreased in the bleeding group.

CAD = coronary artery disease; AMI = acute myocardial infarction; ACS = acute coronary syndrome; CVA = cerebrovascular accident; CVDs = cardiovascular diseases; CHD = congestive heart disease; TGA = thrombin generation assay; CAT = calibrated automated thrombogram; TF = tissue factor; ETP = endogen thrombin potential; VI = velocity index; pM = picomolar.

activates platelets through PAR receptors and the activated platelets are a source of phospholipids to drive coagulation reactions and release of additional procoagulant proteins, such as factor V [6]. Thus, the most suitable biological sample could be PRP [36].

One should also consider the possibility of greater activation of platelets due to the continuous generation of thrombin in vivo, which leads to the consumption of clotting factors available in the plasma and, consequently, a lower potential for thrombin generation in vitro, as argued by Gremmel et al. [52]; this cohort study with 108 participants who underwent angioplasty with stent implantation followed up for 2 years, reported that atherothrombotic events (non-fatal AMI and stroke, and cardiovascular death) occur more frequently among participants with a lower peak of thrombin generation. They also measured the activation platelet through thrombin receptor-activating peptide (TRAP)-6 inducible P-selectin expression and found an inverse correlation between peak thrombin generation and activation platelet [52].

Considering the crucial role of platelets in the development of atherothrombotic events, in addition to other blood cells that influence hemostasis, it is possible that the TGA performed in PRP and in whole blood is much more informative and suitable for the study of diseases in the arterial territory. In this sense, Tripodi [53] has stated that, for example, microparticles, extracellular neutrophil traps (NETs), pro-inflammatory cytokines and other factors circulating in the blood of patients with various clinical conditions can also contribute to hemostatic imbalance. For example, microparticles that originate from endothelial cells, monocytes or platelets are known to carry considerable amounts of tissue factor or negatively charged phospholipids that may contribute to the procoagulant imbalance observed in plasma. NETs can activate platelets and red blood cells, promote thrombus formation and enhance thrombin generation in plasma through the reduction of PC activation by trombomodulin [53]. Thus, the ability of plasma to generate thrombin may not be the only parameter that governs the process, and many known or unknown factors, however existing in the different populations investigated, undoubtedly interfere in a variable way with the results of the studies addressed by us in this brief review, partially justifying their heterogeneity.

Despite the limitations, studies indicate that TGA can be an important tool for understanding the pathophysiological mechanisms of CVDs, understanding the nature and direction of effects of cardiovascular risk factors, and also for cardiovascular risk stratification and mortality prediction [14–16]. Motivated by the potential clinical application of TGA, ISTH members have been developing several studies for the standardization and automation of this technique, in order to propose a unified protocol for performing it [12].

So far, there is no consensus on a TGA parameter that, in isolation, best represents thrombin generation. ETP and peak are the most used parameters, as they are related to the amount of thrombin generated during the reaction, taking into account the pro and anticoagulant forces. Lagtime, time-to-tail and time-to-tail are related to time. However, the evaluation of thrombin generation curve as a whole can better indicate the hemostatic conditions of the individual. The assessment of thrombin generation can be an additional factor in a predictive model to predict CVD in the future. However, it is necessary to understand how the hemostatic system behaves in the face of diseases and in which biochemical interactions the thrombin may be involved, in order to build a solid base of knowledge about the potential for thrombin generation.

6. Conclusions

TGA can be a potentially interesting assay, also in subjects with CVDs, but its usefulness for clinical translation needs better understanding of the underlying determinants of thrombin generation. It could be used for understanding the pathophysiological mechanisms of

CVDs, understanding the nature and direction of effects of the cardiovascular risk factors, and also for stratifying cardiovascular risk [14–16]. This technique is a relatively recent laboratory tool capable of evaluating the functioning of the hemostatic system and has been used in several prospective studies with different purposes. Despite the lack of standardization that still makes its use in clinical practice difficult, the results show that TGA can be an important risk biomarker. However, it is necessary to understand how the hemostatic system behaves in the face of diseases, especially CVDs, and what is the predictive capacity of the TGA. Given the limited knowledge of all biochemical interactions in which thrombin may be involved, building a solid base of knowledge about the potential for thrombin generation and the incidence of CVDs becomes important and deserves attention from the scientific community.

Declaration of competing interest

The authors state that there is no conflict of interest in the development of the study.

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