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Biomarkers of deep venous thrombosis

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Abstract Deep venous thrombosis (DVT), which is associated with pulmonary embolism, is a fatal disease because of its high morbidity and mortality in outpatients and inpatients, especially in hospitalized patients. At the same time, lack of subjective clinical symptoms and objective clinical signs makes the diagnosis complicated. Historically, the primarily imaging modalities, including duplex ultrasound, helical CT scans, and venography, establish the diagnosis of DVT. Currently, both imaging modalities and serology are utilized. These plasma molecules are regarded as the biomarkers of DVT including D-dimer, P-selectin, Factor VIII, thrombin generation, inflammatory cytokines, microparticles, fibrin monomer, leukocyte count and so on. This brief review is used to analyze the contribution of the biomarkers to diagnosis and guidance of therapy for DVT.

Keywords DVT · D-dimer · P-selectin · FVIII · TG · Inflammatory cytokines · MPs · FM · Leukocyte count

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Introduction

Deep venous thrombosis (DVT) is a condition in which a blood clot forms in one or more of the deep veins in your legs. It occurs when bloodstream slows down, vascular wall injures or blood thickens according to Virchow's traid [1]. DVT is a serious condition because a blood clot that has formed in your vein can break loose and travel to your lungs forming pulmonary embolism (PE). However, acute DVT occurs without any noticeable symptoms in about half of all cases, which complicates the diagnosis. Previously, the diagnosis of acute DVT has relied on the primarily imaging modalities. Any suspected patient is requested to subject to compression ultrasound, which was used as the reference method for detection of DVT. Currently, not only imaging modalities but also serology is utilized. These plasma molecules are regarded as the biomarkers of DVT including D-dimer, P-selectin, Factor VIII (FVIII), thrombin generation (TG), inflammatory cytokines, Microparticles (MPs), fibrin monomer (FM), leukocyte count and so on. This brief review is used to analyze the contribution of the biomarkers to the diagnosis and treatment of DVT.

P-selectin

P-selectin is a member of the selectin family of cell adhesion molecules that also includes E-selectin and L-selectin [2], which is stored in granule membrane of unstimulated platelets (α-granules) and endothelial cells (Weibel-Palade bodies) [3]. P-selectin glycoprotein ligand 1(PSGL-1) is the dominant ligand for P-selectin in vivo, which is expressed on the majority of leukocytes and is also found in small amounts on platelets.

During cell activation transmembrane P-selectin is redistributed onto the cell surface and partially released

into the circulation in its soluble form (sP-selectin). It mediates the interaction of stimulated platelets and endothelial cells with leukocytes that express PSGL-1 [4–6]. In the place where P-selectin-PSGL-1 interaction supports leukocyte rolling, P-selectin captures leukocytes from the blood to bring them into contact with the endothelial cell surface on the blood vessel wall [7].

P-selectin-PSGL-1 interaction plays a central role in thrombus formation [8–10]. Palabrica et al. [9] demonstrated that P-selectin can influence fibrin deposition in thrombus. By specifically blocking P-selectin interactions using an antibody against P-selectin, they observed that not only leukocyte adhesion to platelets but also fibrin deposited on a thrombogenic graft in a baboo was inhibited. After administering collagen plus epinephrine to wild type and PSGL-1 knockout mice, Kornél Miszti-Blasius et al. [10] observed that milder thrombocytopenia, less fibrin deposition and lower number of thrombosed blood vessels in PSGL-1-null mice. Thus, it is possible that lack of PSGL-1 may inhibit leukocyte-platelet interactions and reduce the potential of thrombus formation. In summary, inhabitation of the interaction between P-selectin and PSGL-1 was associated with a strong antithrombotic effect. Therefore, targeting P-selectin or its ligand PSGL-1 could provide a potential therapeutic approach for clinical situations. In a meta-analysis, Eduardo Ramacciotti et al. [11] compared the efficacy of P-selectin or its ligand PSGL-1 inhibitors with the low-molecular-weight-heparin enoxaparin for resolution of venous thrombosis in nonhuman primate models. The review suggested that P-selectin antagonism should be further evaluated for the treatment of DVT in nonhuman primate models, by decreasing thrombus burden without causing any bleeding complications and without increasing coagulation times.

In addition to its roles in mediating the binding of platelets and endothelial cells with leukocytes and enhancing fibrin deposition, many studies indicated that P-selectin-PSGL-1 interaction leads to inducing a procoagulant state by triggering formation of leukocyte-derived microparticles [12] and mediating the transfer of tissue factor (TF) to platelets [13].

Recent studies have showed that elevated P-selectin has been implicated as a risk factor for DVT. Rectenwald et al. [14] measured P-selectin in patients with acute DVT confirmed by duplex ultrasound and compared these values with a group of healthy and a group of symptomatic individuals those were negative on duplex ultrasound for DVT. They found remarkably increased sP-selectin concentrations in patients with acute DVT and of predictive value for confirming DVT. This implied the clinical applicability of P-selectin measurements to assess the risk of DVT. Recently, Eduardo Ramacciotti and collaborators, prospectively evaluating the combination of sP-selectin with other biomarkers and clinical characteristics such as D-dimer, C-reactive protein (CRP), MPs and clinical Wells Score (Table 1) in 62 positive and 116 patients with negative DVT, demonstrated that sP-selectin could establish the diagnosis of DVT with a cut point of 90 ng/mL, when combined with the Wells score $\geq$2, with a specificity of 96 % and positive predictive value of 100 %, and could exclude DVT diagnosis with cut points below 60 ng/mL, when combined with the Wells Score <2, with a sensitivity of 99 %, a specificity of 33 %, and a negative predictive value of 96 % [15]. This study suggested that the combination of sP-selectin and Wells score could exclude and confirm the diagnosis of DVT.

Table 1 The original Wells score

Criterion	Score if present
Lower limb trauma or surgery or immobilisation in a plastercast	+1
Bedridden for more than three days or surgery within the last four weeks	+1
Tenderness along deep venous system	+1
Entire limb swollen	+1
Calf more than 3 cm bigger circumference, 10 cm below tibial tuberosity	+1
Pitting oedema	+1
Dilated collateral superficial veins (non-varicose)	+1
Malignancy (including treatment up to six months previously)	+1
Alternative diagnosis as more likely than DVT	−2

Risk Category: low risk $\leq$ 0 points; intermediate risk = 1 or 2 points; high risk $\geq$3 points. Adapt from [114]

One of P-selectin gene (SELP) variants is the single nucleotide polymorphism (SNP) 37674AC (rs6136) that confers a threonine to a proline change in position 715 [16]. Cihan Ay et al. reported that Pro715 carriers had significantly lower sP-selectin concentrations. They also observed a higher proportion of Pro715 carriers among the control individuals than among patients with venous thromboembolism (VTE) [17]. On the other hand, it was reported that there was no association between VTE and Pro715 when the factor V Leiden mutation (FVL) exists [18]. Undas et al. [19] showed that the Pro715 carriers released less P-selectin and with a lower velocity upon injury compared to Thr715 homozygotes in recurrent DVT patients not carrying FVL. In healthy individuals and patients after one DVT, such effect was not observed.

D-dimer

D-dimer is a specific fibrinolysinum-mediated breakdown product of crosslinked fibrin [20]. Thrombin converts

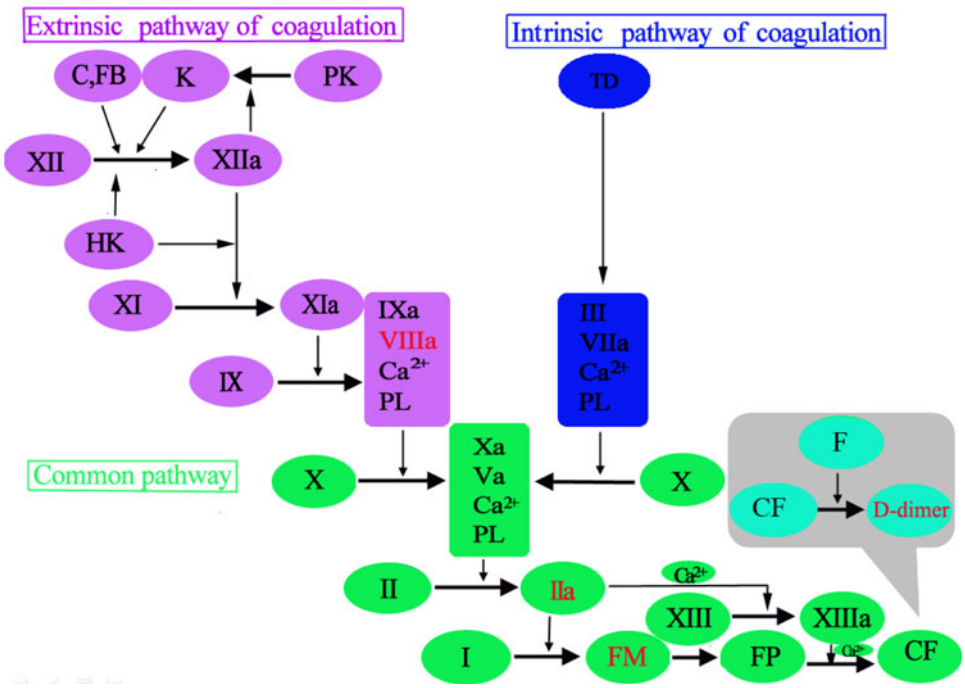

Fig. 1 The extrinsic and intrinsic pathway of coagulation and the formation process of D-dimer. ⟶: Effect of catalysis; ⟶: Direction of change. *Roman numerals* represent the corresponding clotting factor, *C* Collagen, *FB* Foreign body, *K* Kallikrein, *PK* Prekalikrein, *HK* High-molecular-weight Kininogen, *PL* Phospholipid, *TD* Tissue damage, *FM* Fibrin monomer, *FP* Fibrin polymer, *CF* Crosslinked fibrin, *F* Fibrinolysinum

fibrinogen into soluble FM, which then spontaneously polymerizes to form the soluble fibrin polymer. With the presence of calcium, thrombin also activates factor XIII, which crosslinks the fibrin polymer, producing crosslinked fibrin. Subsequently, fibrinolysinum cleavage of the factor XIIIa-mediated crosslinked fibrin produces D-dimer (Fig. 1).

As thrombin and Factor XIII participate in the formation of D-dimer, we suspect it ought to be elevated whenever there is activation of thrombin to form crosslinked fibrin, i.e. DVT. Thereby, numerous published studies concerning D-dimer confirmed whether the conjecture was true. Khaira and Mann evaluated 80 consecutive patients via a clinical diagnosis of DVT, and in 29, DVT was confirmed by venography [21]. Plasma D-dimer levels of these cases had a sensitivity of 96 %, specificity of 40 %, PPV of 48 %, and NPV of 95 % when compared to venography. Thus, the authors concluded that a normal plasma D-dimer level could be used as a test of exclusion for DVT. In other words, D-dimer cannot be the sepcific index for DVT, because it is also elevated in a number of other common clinical situations (liver disease, pregnancy, recent trauma or surgery, cancer, massive bleeding, multiple traumatic injuries and so on [22–25]). Mojca Bozic et al. [26] agreed on the concept. The authors utilized 6 kinds of D-dimer assays to compare their advantages and disadvantages, and demonstrated that all D-dimer assays investigated reliably excluded DVT in those patients without DVT. However, the D-dimer concentration increases with age and its specificity for embolism decreases, which makes the test less useful to exclude PE in older patients [27]. Renee et al. gave us a new D-dimer cut-off value defined as (patient's age × 10) ug/l in patients aged >50 [28].

D-dimer testing, the most frequent use of which is in the evaluation of patients with clinical suspicion of VTE, is now widely employed in clinical practice. Outpatients with elevated levels of D-dimer based on observational studies were proven to be with VTE, obviating the need for investigations such as ultrasound [29, 30]. Furthermore, a prospective study of patients seen in an acute-care setting for serum D-dimer level precludes the need to undergo pulmonary computed tomography (CTA) [31]. Lana et al. thought pulmonary CTA findings positive for acute embolism should be viewed with caution, especially in patient with a serum D-dimer level of ≤1.0 μg/ml [32]. Although some researchists came up with which the modality of angiography carried several negative consequences, cost too much to our patients and the test results were associated with the reporters [33–35], I think this modality has the irreplaceable value in diagnosis of acute PE. Interestingly, Crowther et al. [36] demonstrated D-dimer should not be used to guide diagnostic testing for DVT in critically ill patients. The authors especially highlighted the complete lack of utility of the bedside D-dimer test in critically ill.

Moreover, D-dimer levels play a role in predicting the risk of VTE recurrence and assessment of the duration of therapy for VTE. In 2006, a PROLING study was published on the New England Journal of Medicine to explore the issue [37]. The authors summarized that patients with an abnormal D-dimer level 1 month after the discontinuation of anticoagulation had a significant incidence of

recurrent VTE, which was reduced by the resumption of anticoagulation. Benide et al. indicated that repeated D-dimer testing especially in first 3 months after vitamin K antagonists (VKAs) cessation for a first episode of unprovoked VTE could identify a subgroup of patients with a low risk of recurrence, which might not warrant prolonged anticoagulation [38]. Subjects with repeated normal D-dimer more than 3 months after VKAs cessation and without significant residual venous obstruction did not resume VKAs whereas subjects in whom D-dimer became abnormal over the first 3 months after stopping anticoagulation resumed VKAs. In those patients with an abnormal D-dimer, D-dimer was abnormal in the majority of cases after 3–6 months. They should either continue or resume VKAs for the long term. Recently, an article described that in patients with acute PE elevated D-dimer was associated with increased short-term (within 30 days) and 3 month mortality [39], suggesting the potential of using D-dimer test for risk stratification.

Factor VIII

FVIII is a glycoprotein cofactor, which has been found to be synthesized and released into the bloodstream by the vascular, glomerular endothelium and the sinusoidal cells of the liver [40]. In the circulating blood, it is mainly bound to von Willebrand factor (vWF) to form a stable complex. Under the vivo conditions, thrombin is the only activator of physiological relevance to generate FVIIIa [41]. Upon activation by thrombin, it dissociates from the complex to interact with Factor IXa in the coagulation cascade. It converts Factor IX into Factor IXa, which, in tune, with its cofactor Factor Va, activates more thrombin. Thrombin cleaves fibrinogen into fibrin which polymerizes and crosslinks (using Factor XIII) into a blood clot (Fig. 1).

High FVIII is an independent [42, 43] and a dose-dependent [44] risk factor for both a first and a recurrent VTE. Ted Koster et al. undertook a population-based patients-control study to elucidate the role of the ABO blood group, vWF and FVIII in the process of DVT [43]. In univariate analysis, all of them were correlated with DVT, while only FVIII remained as a risk factor in multivariate analysis. Consequently, it seems likely that blood group and vWF are involved in a common causal pathway of thrombogenesis, and their effects on thrombosis are mediated by FVIII. In 2000, Kraaijenhagen and his co-workers observed that for each 10 IU/dl increased in FVIII, the risk of first VTE increased by 10 %. Furthermore, with each 10 IU/dl increment in FVIII, the risk of recurrent VTE increased by 24 % [44]. Simultaneously, Kyrle et al. also obtained the same conclusion via exploring the relationship between high plasma levels of FVIII and the risk of recurrent VTE [45]. The relation between FVIII and the risk of recurrence was nonlinear. The risk of recurrent thrombosis for individuals with a FVIII level above 90th percentile was 7.4 times higher than subjecets with lower levels. The authors indicated that a high level of FVIII was a cause rather than a consequence of VTE. This concept was also supported by observation that among patients with VTE, high levels of FVIII persisted over time [46, 47].

Currently, some researchers undertook the research of the relations between thrombosis and FVIII in the genetic aspect. Soria et al. represented the first direct evidence that activated protein C resistance (APCR) and FVIII levels, which were major risk factors underlying liability to thrombosis, were jointly influenced by a quantitative trait locus (QTL) on chromosome 18 [48]. Simultaneously, this QTL was an important modulator of an individual's susceptibility to thrombosis. But there were no obvious candidate hemostasis-related genes that might influence the APCR ratio or FVIII levels in the QTL. And so far, little novel mutations or polymorphisms related to FVIII have been identified. More recently, 92714C > G (rs1800291), a SNP encoding the B-domain substitution D1241E has been confirmed, and is significantly associated with FVIII level [49].

Inderijit et al. evaluated the antithrombotic efficacy of the partially inhibitory human monoclonal antibody against FVIII, mAb-LE2E9, in mouse models of inferior vena cava thrombosis and made a conclusion that the antibody could markedly inhibit thrombosis without the risk of overdosing and causing spontaneous bleeding [50]. Recently, an article reported that partial FVIII inhibition yielded similar antithrombotic effects as nearly complete FVIII inhibition whilst avoiding excessive anticoagulation in a mouse model of VTE [51]. These observations may have important implications for the development of efficient, easy, and safe strategies for the prevention and treatment of venous thrombosis.

Thrombin generation

As mentioned above, thrombin is pivotal for the acceleration of the coagulation cascade, because it serves as an activator for platelets, Factor V, and FVIII, and is a critical component of a positive-feedback loop that results in the generation of large amounts of additional thrombin, the conversion of fibrinogen to fibrin, and ultimately, clot formation [41].

Although the measurement of TG has been available since 1953 [52], only recently have assays been developed with which TG can be efficiently measured [53]. By immunologic-based assays, the generation of thrombin can be monitored in the plasma of individual who is suffering the activated coagulation cascade caused by TF and phospholipids, and can be registered in a TG curve. TG can

be expressed in multiple ways, including the lag time (time until thrombin burst appears), the peak TG (the maximal concentration of thrombin formed at a given point in time), and the endogenous thrombin potential (ETP) (the area under the curve).

It has been demonstrated that TG is one of the risk factors for VTE [54], and can be useful as a predictive marker for evaluating thrombosis on an individual basis [55]. Dargaud et al. found that TG was reduced in patients with bleeding tendency, while increased in patients at risk of VTE [56]. Recently, a study explored that both ETP and the peak TG were significantly elevated in patients with a prior history of VTE, and furthermore TG had a significant correlation with FVIII level [57]. Some researchers completed a prospective cohort study to identify patients at low risk for recurrent VTE by measuring the level of TG in plasma [58]. In 2009, Lutsey et al. came to the conclusion that elevated basal peak TG was associated with the risk of recurrent VTE, independently of confirmed VTE risk factors [59]. Interestingly, Vlieg et al. deemed that elevated ETP was associated with an increased risk of a first DVT but not with the risk of recurrence [60].

Seqers and co-workers investigated whether the TG assay could detect changes in the haemostatic balance associated with common genetic variation affecting the level or function of coagulation factors and inhibitors [61]. TG parameters were increased by F5 Leiden, F2 G20210A and F2 A19911G, while decreased by FGA A1069G, F10 IVS2 C + 517G, F12 C-46T, TFPI T-287C and TFPI IVS7 T-33C. These results indicated that the TG was sensitive to genetic variation in haemostasis-related genes and might be an intermediate phenotype for VTE, which made it a promising tool to identify novel genetic risk factors of VTE.

Conversely, some investigators thought TG parameters alone were inappropriate for the exclusion of DVT and only in the elderly, might the current algorithm of exclusion of DVT improve markedly by the addition of the lag time results of ETP [62]. As mentioned above, a normal D-dimer level can exactly exclude DVT in subjects. So we hypothesize whether the combination of D-dimer and TG can increase the sensitivity and specificity, PPV, and NPV for diagnosing or excluding VTE.

Inflammatory cytokines

Increasing evidence suggests a role for inflammatory markers such as CRP and interleukin (IL)-1β, 6, 8, 10 in VTE. Inflammatory cytokines may influence the expression of TF, an initiator of the extrinsic pathway of coagulation, thus providing a trigger that may lead to thrombotic disease [63]. Lab studies have recently demonstrated that increased CRP levels had a significant effect on the subsequent of VTE [64, 65]. However, in a prospective study that has examined the predictive value of plasma CRP level for the development of VTE, Tsai et al. found that there was no relationship between base line CRP levels and the subsequent development of VTE [66]. In addition, another four studies evaluated the potential role of CRP in the diagnosis of VTE [67–70]. Fox et al. combined the data from the four studies and yielded a pooled weighted sensitivity of 77 % and specificity of 66 %.Thus, plasma CRP level, used alone, does not appear to be useful to diagnose DVT [71].

Reitsma and Rosendaal exhibited a probable association between VTE and several other markers of inflammation by measuring plasma tumor necrosis factor (TNF)-α, IL-1β, IL-6, IL-8, IL-10 and IL-12p70 levels in a case–control study for venous thrombotic disease [72]. TNF-α, IL-6 and IL-8 levels were found to be risk determinants for VTE. Conversely, the risk for the anti-inflammatory cytokine IL-10 tended to be decreased. For IL-12p70, there was no association with VTE, while the association was weak for IL-1β levels. Pavel Poredos et al. also found that patients with idiopathic VTE had not only increased levels of pro-inflammatory cytokines such as IL-6 and IL-8 but also significantly decreased levels of anti-inflammatory interleukins, particularly IL-10 [73]. In their study, they also showed that endothelial function was deteriorated in patients with VTE. They hypothesized that the imbalance between pro- and anti-inflammatory activities promoted thrombus formation not only through its involvement in the coagulation cascade but also through deterioration of endothelial function. However, in another two studies, there was no statistically significant association of VTE with the levels of inflammatory cytokines [74, 75].

Based on the association between inflammation and coagulation it is possible that polymorphisms in genes encoding for proteins involved in inflammation may influence susceptibility towards VTE. Beckers et al. investigated the occurrence of 49 inflammation-related gene polymorphisms in both the patients with VTE and control groups. SNPs of IL-1A, IL-4, IL-6 and IL-13 were found to be associated with the occurrence of VTE [76]. VTE was significantly less frequent in patients with the CT genotype and carriers of the T allele of the −899 CT polymorphism (rs17561) in the IL1A gene, as compared to the control group. In addition, Gender differences were observed for SNPs in IL-4, IL-6 and IL-13.For instance, the IL-6-174-CC (rs1800795) genotype was associated with VTE in male patients. Robert et al. evaluated potential associations of 51 polymorphisms from 32 inflammation-related genes with risk of incident VTE in a prospective cohort of 22,413 white women followed over a 10 year period. They found variation at rs1143634 in the IL-1B gene was associated with a reduced risk of idiopathic VTE

while variation at rs1800872 in the IL-10 gene was associated with increased risk [77]. However, in a case–control study, IL-6 -174GC, IL-8 -251AT and MCP-1 -2518AG SNPs did not influence the risk of VTE and the cytokine levels [78].

A study by Downing et al. showed that neutralization of IL-10 increased inflammation and thrombosis, while supplementation with exogenous IL-10 demonstrated a decrease in inflammation and thrombus formation. These suggested that IL-10 could be used as a therapeutic agent in the treatment of VTE [79].

Microparticles

MPs, which are by definition between 0.1 and 1.0 μm in size, are small membranous vesicles released from the plasma membranes of platelets, leukocytes, red cells and endothelial cells in response to apoptosis or cellular activation [80, 81]. MPs are detected and characterized on the presence of surface antigens from their respective parental cells [82]. Historically, MPs were consistent with "cellular dust" without any biological function [83]. Recently, it has been hypothesized that MPs play a role in inflammation, coagulation and vascular function [84].

Elevated levels of MPs are encountered in diseases with vascular involvement and hypercoagulability, where they appear indicative of a poor clinical outcome [85], and had been documented in the blood of patients with VTE [86]. There are two animal researches both elucidating a crucial role for MPs in thrombosis from the opposite sides. One ascertained platelet CD36 mediated the interactions between endothelial cells and derived MPs and ultimately contributed to thrombosis in mice [87], the other showed that stimulation of monocytes with a P-selectin and immunoglobulin chimera increased the number of circulating monocyte MPs and restored hemostasis in hemophilia A mice, leading the author to propose that MPs could be used to treat patients with hemophilia [88]. Moreover, MPs are the main carriers of circulating TF, the principal initiator of intravascular thrombosis. Ramacciotti et al. illuminated that MP concentration and TF activity directly correlated at a highly significant level in an experimental mouse of venous thrombosis [89]. TF-positive MPs may ultimately prove to be a useful biomarker to identify patients at risk for thrombosis.

Recent evidence suggests MPs carry RNA [90, 91] and DNA [91]. In vitro, MPs contain both nuclear and cytoplasmic DNA and RNA, especially low molecular weight RNA in the size range of microRNA [90]. What's more, MPs play an important role in transferring mRNA from endothelial progenitor cells to endothelial cells to activate an angiogenic program [91]. We can speculate that DNA and RNA included in MPs may influence thrombosis through a horizontal transfer of genetic mass between platelets and endothelial cells.

Fibrin monomer

The thrombotic response involves a variety of procedures, for example platelet activation, enhanced activity of coagulant system, fibrin formation [92]. FM is the product of thrombin-induced proteolysis of fibrinogen (Fig. 1). So Vogel suggested that quantitative FM be a valuable diagnostic tool for the early diagnosis of postoperative DVT [93]. During the test, the FM assay had a specificity of 73.2 % and sensitivity of 91.7 %. Simultaneously, all FM-positive DVT-patients had pathological FM-values at least the day prior to the clinical manifestation of thrombosis. Reber et al. couldn't agree to this opinion. They found that serial FM measurements were unable to predict or exclude DVT in asymptomatic patients undergoing total knee arthroplasty [94].

What's the difference between D-dimer and FM? Both of them are fibrin-related markers. D-dimer is a fibrinolysinum-mediated breakdown product of crosslinked fibrin in the post-thrombotic state [20]. Contrarily, FM is produced by thrombin-mediated cleavage of fibrinogen in a hypercoagulable state [41]. Therefore, D-dimer could be regarded as a post-thrombotic marker while FM could be regarded as a pre-thrombotic marker [95]. One study had evaluated whether FM was aid to D-dimer analysis when excluding DVT in symptomatic outpatients. Compared with just D-dimer analysis, simultaneous D-dimer and FM determination provided a more valuable approximation for DVT [96]. And Park et al. evaluated the diagnostic performance of FM for disseminated intravascular coagulation (DIC) in comparison with D-dimer [97], and found that FM had higher sensitivity, specificity, PPV, and NPV than D-dimer for differentiating overt DIC from non-DIC.

Leukocyte count

Blood leukocytes can be stimulated by a variety of agents to develop potent procoagulant activity able to initiate the extrinsic pathway of blood clotting [98]. It has also been demonstrated that leukocyte adhesion and transmigration are the early events in the initiation of DVT [99]. Reyers et al. designed experiments to evaluate the role of platelet and leukocyte number in experimental VTE in rats. They found that in normal animals the leukocyte count was significantly raised after ligature depending mainly on the duration of the stasis. Stoffel et al. retrospectively evaluated the association of leukocyte counts and thrombosis in three cohorts of 100 patients each undergoing intensive cytoreductive treatment for haematological malignancy. The results confirmed a strong association of leukocytosis

with development of thrombosis [100]. However, two recent studies on patients with essential thrombocythemia (ET) showed no statistically significant association of thrombosis with leukocyte count [101, 102].

The most powerful prediction of thrombosis by leukocyte count seems limited to patients with ET at low-risk of thrombosis (i.e. below 60 years of age, no prior thrombosis). Passamonti et al. evaluated the impact of the increase in leukocyte count over time on the risk of thrombosis. They demonstrated that low-risk ET patients with an increase in leukocyte count in the two years from diagnosis had higher risk of thrombosis than patients with stable leukocyte counts [103].

Discussion

As mentioned above, there are a lot of biomarkers identified for diagnosis of or treatment for DVT. Actually, these biomarkers can be divided into two categories from pathobiology of DVT or thrombotic disease. One is coagulation markers, such as D-dimer, FVIII, TG, and FM, while the other is inflammatory markers, including P-selectin, inflammatory cytokines, MPs and leukocyte count. And what's the pathologic function of these biomarkers during the formation process of DVT (Table 2)?

Although all of these biomarkers theoretically have connection with the pathobiology of DVT, each of them plays a different role in diagnostic or prognostic purposes of DVT. D-dimer is now extensively used for diagnosis of thrombotic diseases. In fact, only a normal plasma D-dimer level can be used as a test of exclusion for DVT [21] (Table 3). Whether others can be employed in diagnosis of DVT alone during clinical practice is uncertain because study findings are inconsistent. It may be due to ethnic differences, environmental differences, different selection criteria, different research methods, different statistical methods, and so on. By combining one biomarker with another [96] or clinical score [15], the PPV of DVT can be elevated markedly (Table 4). The combination of sP-selectin and Wells score could exclude and confirm the diagnosis of DVT when sP-selectin <60 ng/ml plus Wells Scores <2 and sP-selectin ≥ 90 ng/ml plus Wells score ≥ 2, respectively. All of the biomarkers can be utilized for evaluating individual at the risk of DVT, especially D-dimer, FVIII, and TG. Persistent high levels of D-dimer, FVIII, and TG prognosticate that individuals are at risk of not only first DVT, but also recurrent DVT [37, 38, 44, 46, 59, 60].

Two prospective studies involving patients with VTE showed that D-dimer levels was responsible for predicting the risk of VTE recurrence and subsequent assessment of the duration of therapy for VTE [37, 38]. Repeated D-dimer testing especially in first 3 months after VKAs withdrawal for first VTE could identify a subgroup of patients with an either low or high risk of recurrence (Table 5). And so far, analogous studies that referred to the

Table 2 The source of biomarkers and their pathobiology of DVT

Biomarkers	Source	Pathobiology of DVT
D-dimer	A fibrinolysinum-mediated breakdown product of crosslinked fibrin [20].	Thrombin and Factor XIII participate in the formation of D-dimer in the post-thrombotic state [95].
P-selectin	A transmembrane protein of endothelial cells and platelets [2].	The interaction between p-selectin and PSGL-1 can lead to mediating the binding of platelets and endothelial cells with leukocytes [4–7], enhancing fibrin deposition [8–10], triggering formation of leukocyte-derived microparticles [12], and mediating the transfer of TF to platelets [13].
FVIII	A glycoprotein cofactor, synthesized and released into the bloodstream by the vascular, glomerular endothelium and the sinusoidal cells of the liver [40].	FVIII participates in the intrinsic pathway of coagulation and converts Factor IX into Factor IXa [41].
TG	A prothrombinase-mediated breakdown product of prothrombin [41].	It serves as an activator for platelets, Factor V, and FVIII and converts fibrinogen to fibrin [41].
Inflammatory cytokines	Low-molecular-weight proteins released by leucocytes, endothelial cells, fibroblasts, smooth muscle cells, and so on [104, 105].	Inflammatory cytokines influence both endothelial function and the expression of TF [63, 73].
MPs	Small membranous vesicles released from plasma membranes of platelets, leukocytes, red cells and endothelial cells [80, 81].	MPs play roles in inflammation, coagulation and vascular function [84] and are the main carriers of circulating TF [89].
FM	A thrombin-mediated breakdown product of fibrinogen [41].	FM participates in the both intrinsic and extrinsic pathway of coagulation [95].
Leukocyte count	Posterity of hemopoietic stem cells.	Leukocyte adhesion and transmigration are the early events in the initiation of DVT [98, 99].

Table 3 Overall accuracy indices (sensitivity, specificity, NPV, and PPV) of DVT for all D-dimer tested variables

Assay	Cut-off (μg/l)	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)
A	≥137	100 (100–100)	78 (71–85)	100 (100–100)	74 (67–81)
B	≥876	100 (100–100)	73 (66–81)	100 (100–100)	69 (62–77)
C	≥114	100 (100–100)	22 (15–29)	100 (100–100)	44 (35–52)
D	≥344	100 (100–100)	61 (53–69)	100 (100–100)	61 (53–69)
E	≥200	100 (100–100)	60 (51–68)	96 (93–99)	60 (52–68)
F	≥250	100 (100–100)	51 (43–60)	98 (95–100)	55 (47–63)

Results are given with 95 % confidence interval. Assays A and B were ELISAs (Dade Behring and Diagnostica Stago, respectively), assays C and D automated turbidimetric assays (Dade Behring and Biopool, respectively), assay E qualitative erythrocyte agglutination assay (Agen Biomedical) and assay F semiquantitative latex agglutination assay (Biopool). Adapted for Table 3, Bozic et al. [26]

Table 4 Summary of sensitivity, specificity, PPV, NPV in which a biomarker plus clinical score is the independent variable

Variables	Cut-off	Sensitivity (100 %)	Specificity (100 %)	NPV (100 %)	PPV (100 %)
sP-selectin + Wells score	≥90 ng/ml + ≥2	33	95	70	100
sP-selectin + Wells score	<60 ng/ml + <2	99	33	96	47

Results are given with 95 % confidence interval. sP-selectin were tested by ELISAs (R&D Systems Inc) and Wells Score were referred to Table 1. Adapted for Table 4, Ramacciotti at al. [15]

Table 5 Patients with a first episode of VTE in whom D-dimer was different at the moment of VKAs cessation and in 3 months after VKAs cessation had different rate of VTE recurrence and were given different treatment

The level of D-dimer at the moment of VKAs cessation	The level of D-dimer in 3 months after VKAs cessation	The rate of VTE recurrence (%)	Treatment
Normal	Normal	2.9	Do not resume VKAs
Normal	Abnormal	11.1	Resume VKAs
Abnormal	Abnormal	27	Continue or resume VKAs

Results are given with 95 % confidence interval. Adapt for Table 3, Cosmi et al. [38]

Table 6 SNPs of biomarkers as mentioned above were found to be associated with VTE

Biomarkers		SNPs	Reference
P-selectin		rs6136	[16–19]
FVIII		rs1800291	[49]
Inflammatory cytokines	IL-1	rs17561	[76]
		rs1143634	[77]
	IL-6	rs1800795	[76]
	IL-10	rs1800872	[77]

relation between other biomarkers and the duration of therapy for VTE have not been published.

Genetic factors play more or less roles in etiopathogenesis of nearly all the diseases and so the thrombosis. Large amounts of studies reported genetic causes of thrombosis were deficiencies of antithrombin, protein C, and its cofactor protein S [106–108], Factor V Leiden [109], prothrombin 20210A mutation [110], blood group [111], hyperhomocysteinemia [112], and so on. As mentioned above, SNPs of P-selectin, FVIII, and inflammatory cytokines are identified to be associated with the occurrence of VTE (Table 6). In other words, these biomarkers are more likely causes rather than consequences of VTE. In sum, these biomarkers certainly have effect on etiopathogenesis of VTE.

Conclusion

Currently, contrast venography is the most reliable way of diagnosing DVT [113], while it cannot be routinely used for screening purposes because of their uncomfortable invasion and complex procedures. Therefore, biomarkers with high sensitivity and specificity are desirous of being identified for screening and early diagnosis of thromboembolism. However, all of these biomarkers play limited roles in prediction for DVT. Although study findings have been inconsistent, we should bear in mind that all of these

biomarkers have partial functions on the routine clinical use of DVT. A novel way was provided to utilize the biomarkers [15, 96], which is evaluating the incremental usefulness of one biomarker for DVT screening in combination with another or clinical score. At the therapy aspect, D-dimer and FVIII have been proven that they have value in decision on the duration of anticoagulation in DVT patients. P-selectin, FVIII and IL-10 bring us the hope that the inhibitors of these biomarkers or anti-inflammatory cytokines may provide us a new choice when dealing with the boring thrombosis. Genetic factors may be essential for the pathogenesis of DVT. Further investigations, including full genome sequencing and genome-wide association studies, may help to elucidate the mechanisms underlying the risk of thrombosis and ultimately may lead to hilastic and therapeutic strategies that would reduce morbidity and mortality of DVT.

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