# Procoagulant Activity in Hemostasis and Thrombosis: Virchow's Triad Revisited

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## Abstract

Virchow's triad is traditionally invoked to explain pathophysiologic mechanisms leading to thrombosis, alleging concerted roles for abnormalities in blood composition, vessel wall components, and blood flow in the development of arterial and venous thrombosis. Given the tissue-specific bleeding observed in hemophilia patients, it may be instructive to consider the principles of Virchow's triad when investigating mechanisms operant in hemostatic disorders as well. Blood composition (the function of circulating blood cells and plasma proteins) is the most well-studied component of the triad. For example, increased levels of plasma procoagulant proteins such as prothrombin and fibrinogen are established risk factors for thrombosis, whereas deficiencies in plasma factors VIII and IX result in bleeding (hemophilia A and B, respectively). Vessel wall (cellular) components contribute adhesion molecules that recruit circulating leukocytes and platelets to sites of vascular damage, tissue factor, which provides a procoagulant signal of vascular breach, and a surface upon which coagulation complexes are assembled. Blood flow is often characterized by two key variables: shear rate and shear stress. Shear rate affects several aspects of coagulation, including transport rates of platelets and plasma proteins to and

from the injury site, platelet activation, and the kinetics of fibrin monomer formation and polymerization. Shear stress modulates adhesion rates of platelets and expression of adhesion molecules and procoagulant activity on endothelial cells lining the blood vessels. That no one abnormality in any component of Virchow's triad fully predicts coagulopathy *a priori* suggests coagulopathies are complex, multifactorial and interactive. In this review, we focus on contributions of blood composition, vascular cells, and blood flow to hemostasis and thrombosis, and suggests cross-talk among the three components of Virchow's triad is necessary for hemostasis and determines propensity for thrombosis or bleeding. Investigative models that permit interplay among these components are necessary to understand the operant pathophysiology, and effectively treat and prevent thrombotic and bleeding disorders.

## Introduction

Abnormalities in blood coagulation are the leading cause of death world-wide, with treatment costs estimated at more than $250 billion and projected to more than triple to $818.1billion by 2030.[1] In addition to inherited bleeding and thrombotic disorders, therapeutic approaches to manage hemorrhagic and thrombotic episodes are expected to paradoxically increase the incidence of thrombotic and bleeding events, respectively. For example, while the hemostatic agent recombinant factor VIIa is highly effective at minimizing bleeding in hemophilic patients with inhibitors, its off-label use in nonhemophilic patients has been associated with thrombosis.[2] Similarly, whereas the recently approved antithrombotic dabigatran exhibits improved safety and efficacy compared to warfarin, there is currently no rapid reversal agent, which may leave patients prone to bleeds.[3] Understanding mechanism(s) of blood coagulation and its associated disorders will allow design of targeted, and therefore safer and more effective, therapeutics to treat both bleeding and thrombosis.

## Tissue factor (TF), thrombin, and fibrin(ogen) in clot formation and stability

Procoagulant activities have been traditionally separated into extrinsic, intrinsic, and common pathways; however, the acknowledgement that thrombin generation must be localized to a site of injury, as well as complex presentation of certain factor deficiencies, has led to the conceptual integration of these pathways.[4] Briefly, coagulation is initiated via extrinsic activity after exposure of cell-derived TF, formation of the factor VIIa/TF complex, and conversion of factor X to factor Xa. Thrombin generation is subsequently augmented by intrinsic (factors XI, IX, and VIII-dependent) activities. Though both the extrinsic (factor VIIa/TF) and intrinsic (factors IXa/VIIIa) tenase complexes produce factor Xa, the relative contributions of these activities are dictated, in part, by the local TF concentration and type of cell surface supporting enzyme/cofactor complex assembly (discussed below). Cellular and plasma-dependent mechanisms culminate in prothrombinase complex (factors Xa, Va, and prothrombin) assembly, and production of the enzyme thrombin. Most current antithrombotic agents target one or more of the active enzymes generated during the clotting cascade, including factors Xa, IXa, and thrombin.

Proteolytic conversion of circulating, soluble fibrinogen to an insoluble fibrin meshwork involves thrombin-mediated cleavage of N-terminal peptides from fibrinogen, end-to-end polymerization of fibrin monomers to protofibrils, and lateral aggregation of protofibrils to fibers. This sequence of events has been extensively studied and reviewed.[5–7] Fibrin's remarkable biophysical characteristics make it extraordinarily suited to provide structural support to the clot; individual fibers can be strained more than 330% without rupturing.[8] As such, fibrin is an effective therapeutic target for both minimizing bleeding in hemostatic

disorders[9], and dissolving intravascular thrombi in myocardial infarction, ischemic stroke, and deep vein thrombosis.[10–12]

Determinants of fibrin network characteristics have been almost entirely studied in purified systems or in platelet-poor plasma. These studies have shown that the conditions under which fibrinogen is converted to fibrin determine the fiber thickness, branching, and network density of the resulting clot. These conditions include the local pH, ionic strength, and concentrations of calcium, polyphosphate, fibrin(ogen)-binding proteins (*e.g.* factor XIII), thrombin, and fibrinogen present during fibrin formation.[13–19] Of these, the influences of thrombin, fibrinogen and factor XIII on fibrin structure and function have been the best characterized. At a constant fibrinogen concentration, low thrombin concentrations produce coarse, unbranched networks of thick fibrin fibers, whereas high thrombin concentrations produce dense, highly branched networks of thin fibers.[15,16] Similarly, at a constant thrombin concentration, increasing the fibrinogen concentration produces denser, highly-branched fibrin networks.[16–18] The structural composition of a fibrin clot is important because the structure determines its biochemical and mechanical properties (reviewed in [20]). In general, coarse networks have a lower elastic modulus (ability to undergo elastic deformation, "stress/strain") and increased susceptibility to fibrinolysis, whereas dense networks have a higher elastic modulus and are relatively resistant to fibrinolysis.[18,21,22] Factor XIII activity provides additional mechanical and biochemical support for the fibrin network by cross-linking adjacent chains, as well as inhibitors ($\alpha$2-antiplasmin) to the clot.[19,23]

## Pathophysiologic mechanisms in thrombosis and bleeding

Although the origin is highly controversial[24], the concept of concerted pathogenic roles for the triad of abnormalities in blood composition, vessel wall function, and blood flow/shear in venous thrombosis/thromboembolism is typically attributed to Rudolph Virchow in the 1700s.[25] Regardless of its controversial origins, the conceptual integration of these functions has considerably advanced understanding of the pathogenesis of thrombosis as well as bleeding (Figure 1). Specific components of Virchow's triad are illustrated in Figure 2. Abnormal levels of pro- and anticoagulant proteins[26–44], thrombin generation[39–45], clotting factor activity[46] or resistance to inactivation[47,48], markers of vascular cell damage or activation[49,50], and fibrinolysis inhibitors[51,52], have been correlated with venous and/or arterial coagulopathies in case reports and small and large epidemiologic studies. That no one abnormality predicts coagulopathy *a priori* highlights the complex, interconnected pathways in which these components regulate coagulation.

Arterial thrombosis is typically associated with atherosclerotic plaque rupture. This pathogenic process results in exposure or release of subendothelial cells and procoagulant material (e.g., TF, collagen) from within the plaque and activation and aggregation of platelets. The growing thrombus increases the degree of stenosis which can result in extremely high shear rates (up to 70,000 s$^{-1}$) within the stenotic region.[53] In some cases, depending on stenosis geometry and location in the vasculature, turbulent flow may develop downstream of the stenosis. Ultimately, platelet accumulation and fibrin deposition produces an occlusive platelet-rich intravascular thrombus (Figure 3).

In contrast, venous thrombosis/thromboembolism is typically associated with plasma hypercoagulability and thought to be triggered by expression of procoagulant activity on intact endothelium from inflammation and/or stasis/reduced blood flow resulting from prolonged immobility. Venous clots have regions or layers showing substantial erythrocyte incorporation (Figure 4).

Given the tissue-specific bleeding (predominantly joints and muscles) observed in hemophilia patients[54–56], it may also be instructive to consider the principles of Virchow's triad when interrogating mechanisms operant in hemostatic disorders. Hemostasis is the response to bleeding resulting from transection of the full thickness of the vessel wall and extravasation of blood into the extravascular space. Hemophiliacs rarely bleed into tissues with high subendothelial/extravascular TF expression, suggesting relative contributions from blood composition (levels of circulating coagulation factors) and tissue-specific coagulant activity (e.g., local TF or thrombomodulin expression) dictate hemostatic potential at an injury site.

Predicting thrombotic or hemorrhagic events and devising targeted approaches for minimizing these events requires thorough consideration of mechanisms that promote these pathologies. This review focuses on the procoagulant contributions of blood composition, vasculature, and flow/shear to hemostasis and thrombosis.

### Contributions of plasma procoagulant activity in thrombosis and bleeding

Likely due to the ease of obtaining blood for *ex vivo* experiments, blood composition (circulating blood cells and plasma proteins) is the best-characterized facet of Virchow's triad. Techniques to evaluate the isolated effects of abnormal plasma clotting factor levels in purified assays have given way to technologies that measure the global hemostatic potential of blood and plasma. These newer techniques permit translation of changes in individual factor levels to net changes in thrombin generation and clot formation and stability.

Deficiencies in either factor VIII or IX (hemophilia A or B, respectively) are well-characterized hypocoagulabilities in which patients produce a primary platelet plug in response to vascular injury, but typically re-bleed hours to days after injury. *In vitro* studies show hemophilic conditions result in reduced thrombin generation, with a prolonged onset and lower thrombin generation rate and peak level.[39,41–43] This abnormal thrombin generation pattern causes deficiencies in fibrin production, fibrin network density and permeability, and susceptibility of the fibrin network to fibrinolysis.[38–42,57–59] Both replacement and bypassing strategies used to treat and prevent bleeding in hemophilia improve thrombin generation variables and accelerate and stabilize the resulting fibrin network.[40,42,58,60]

The role(s) of elevated plasma factor levels (hypercoagulabilities) on clotting are less well understood. Elevated prothrombin levels, typically associated with a glycine to arginine mutation at residue 20210 (G20210A), increase risk of venous thrombosis.[26] The mechanism for this correlation is currently unclear. Patients with the G20210A mutation do not have elevated levels of the prothrombin cleavage product fragment 1.2, suggesting coagulation is not activated at baseline.[61] However, *in vitro* assays with reconstituted systems and patient plasma suggest once coagulation is triggered, hyperprothrombinemia increases the rate and peak of thrombin generation.[61–65] This apparent paradox may be reconciled by the hypothesis that low levels of thrombin activation are functionally anticoagulant because thrombin in complex with thrombomodulin activates negative feedback pathways to limit coagulation, whereas the *in vitro* assays are triggered with higher TF concentrations that overwhelm anticoagulant mechanisms and promote higher procoagulant activity. In the *in vitro* assays, increased thrombin generation results in the formation of fibrin clots composed of a fine network of thin fibrin fibers[63] and increased activation of the thrombin-activable fibrinolysis inhibitor (TAFI)[66], mechanisms that have both been correlated with increased thrombosis risk. Thus, although still to be demonstrated *in vivo*, these data suggest a mechanism correlating elevated prothrombin, thrombin generation, and clot stability.

Abnormalities in fibrin(ogen) synthesis, formation and function are implicated in both bleeding and thrombotic disorders. More than 500 fibrinogen gene mutations have been identified that result in expression of low (afibrinogenemia) or abnormally functioning (dysfibrinogenemia) fibrinogen chains. In addition, posttranslational modification (e.g., oxidation, nitration, homocysteinylation, and glycation) of circulating fibrinogen has been implicated as an "acquired dysfibrinogenemia" in prothrombotic disorders, including acute coronary syndrome, diabetes, hyperhomocysteinemia, and thrombosis associated with acute cigarette smoke exposure, presumably because it alters fibrinogen cleavage, incorporation of fibrin into the fibrin network, and/or fibrin network stability[67]. Although an elevated fibrinogen level (hyperfibrinogenemia) is a well-established risk factor for both arterial and venous thrombosis[29–35,49,68], its etiologic role has been controversial. Transgenic mice that over-express murine fibrinogen show elevated fibrin degradation products (D-dimer) and spontaneous fibrin deposition in the spleen, but only marginal shortening of the time to 75% occlusion after 20% ferric chloride ($FeCl_3$) application to the carotid artery.[69–71] Using a different hyperfibrinogenemia model in which wild type mice were infused with human fibrinogen, we showed that hyperfibrinogenemia shortens the time to occlusion after application of 10% and 5% $FeCl_3$ to the carotid artery and saphenous vein, respectively.[18] Furthermore, hyperfibrinogenemia increases thrombus fibrin content, promotes faster fibrin formation, and increases fibrin network density, strength, and stability. Importantly, hyperfibrinogenemia increases resistance to pharmacologically induced thrombolysis *in vivo.* These data demonstrate a direct, etiologic link between hyperfibrinogenemia, thrombosis, and thrombolysis in acute settings.[18]

## Contributions of cellular procoagulant activity in thrombosis and bleeding

Vascular cells contribute at least two essential functions during coagulation. First, cellular adhesion molecules including P-selectin glycoprotein ligand-1 (PSGL-1) exposed during vascular injury recruit leukocytes and platelets to sites of vascular damage[72]. Second, accumulation of these cells, as well as exposure of active cellular TF (normally sequestered from blood in healthy humans) provides a procoagulant signal of vascular breach and a surface upon which to assemble procoagulant complexes. Evidence suggests extravascular TF exists in complex with factor VIIa[73], such that vascular injury exposes the fully formed factor VIIa/TF complex, enabling immediate activation of factor X to factor Xa. Subsequent assembly of the prothrombinase complex (factors Xa, Va, and prothrombin) takes place on the negatively charged lipid (phosphatidylserine) surface provided by TF-bearing cells and activated platelets. Cells also provide a site for factor XI(a) binding and activation of factor IX, as well as assembly of the intrinsic tenase complex (factors IXa, VIIIa, and X) to augment thrombin generation.

We and others have directly compared the procoagulant activity of intravascular and extravascular cells and their ability to support fibrin formation. Whereas quiescent intravascular cells have little to no TF activity, extravascular cells (e.g., fibroblasts and smooth muscle cells) express considerable TF and are primed to trigger coagulation upon contact with blood. Because thrombin dictates fibrin network formation and network properties, the highly procoagulant extravascular cells support the rapid production of a dense fibrin network that is relatively resistant to fibrinolysis.[74–76] Such networks stabilize the primary platelet plug at a site of vascular injury. The relative ability of extravascular cells to trigger clotting in blood escaping from a ruptured vessel can be implicated in tissue-specific hemostasis. Vascular breach into a site of high TF activity would not require an intact intrinsic pathway; thrombin generation could take place entirely via the TF-driven extrinsic pathway. In contrast, vessel disruption into tissues with lower extravascular TF expression would necessitate intrinsic pathway activity to augment thrombin generation and promote stable fibrin formation. For example, hemophilia patients frequently bleed into

muscles and joints. Although this presentation has been related to physical stress placed on these tissues[54–56], hemophilia patients do not typically bleed into other tissues that endure high physical activity such as the heart. These observations suggest that attributing bleeding solely to physical stress oversimplifies the pathology. Cardiomyocytes express high levels of TF, which can promote substantial thrombin generation independently of intrinsic factors VIII and IX, suggesting tissue-specific extrinsic activity can compensate for reduced intrinsic activity. This scenario illustrates the required integration of two components of Virchow's triad, blood and tissues, in maintaining hemostasis.

Extravascular TF expression/activity is implicated in arterial thrombosis resulting from atherosclerotic plaque rupture. The amount of TF found in atherosclerotic plaques in humans is positively correlated with the thrombogenicity of the lesions after plaquerupture.[77] The importance of TF in the progression of arterial thrombosis is underscored by observations that treatment of human plaques with TF pathway inhibitor significantly reduces adhesion of both fibrinogen and platelets to the plaque, indicating the thrombotic potential of atherosclerotic lesions may be decreased by inhibiting TF-dependent procoagulant activity.[78]

Inappropriate expression of intravascular procoagulant activity is hypothesized to trigger venous thromboembolism (VTE). Proinflammatory mediators including bacterial lipopolysaccharide, and the cytokines interleukin-1β and tumor necrosis factor-alpha (TNF-**α**), induce procoagulant activity (thrombin generation) in cultured endothelial cells via *de novo* TF synthesis and decreased thrombomodulin expression in a time- and dose-dependent manner.[18,76,79,80] TNF-**α**-stimulated, cultured endothelial cells support robust fibrin formation.[76] Laser-induced injury of cultured endothelial cells has also been shown to induce procoagulant activity and fibrin formation, although the time course of those experiments suggests TF activity is released from intracellular stores and is not synthesized *de novo*.[81] *In vivo*, endothelial cells residing in different vascular beds (e.g., aorta *versus* pulmonary microcirculation) have different phenotypes. Even within a given vessel, endothelial cells vary in their expression of coagulation proteins. Brooks *et al*. elegantly showed increased expression of thrombomodulin and endothelial cell protein C receptor, and decreased expression of von Willebrand factor in the valve sinus relative to the immediately adjacent vein lumen in saphenous veins harvested during cardiac bypass surgery.[82] Although these studies convincingly show vascular bed-specific expression of anticoagulant proteins on endothelial cells, evidence for endothelial cell TF expression *in vivo* remains controversial.

Vascular bed-specific function is best illustrated clinically by the specific presentations of thrombosis in either arterial or venous circulation, but rarely both. Additionally, risk factors strongly associated with arterial thrombosis (e.g., hypertension, diabetes mellitus, smoking, hypercholesterolemia) only modestly increase risk of venous thrombosis/thromboembolism[83], and meta-analysis suggests history of unprovoked VTE only slightly increases (~1.5-fold) risk of arterial cardiovascular events over long-term followup.[84] Because any abnormality in the blood is present systemically, these events reflect local abnormalities in vascular bed-specific pro- or anticoagulant activity and/or blood flow (discussed below). Vascular bed-specific activities are further illustrated by animal models of coagulation dysfunction that show bleeding and/or fibrin deposition in specific tissues. For example, mice with partial TFPI deficiency and decreased thrombomodulin function demonstrate fibrin deposition in the brain and liver, but not other tissues.[85] These observations suggest unique regulatory mechanisms are present in specific tissues.

Neither "cell" nor "plasma protein," cell-derived microparticles express cell-specific markers and retain procoagulant properties derived from their parent cell[86,87], and can

circulate and evade normal spatial restrictions on cellular procoagulant activity[75]. Although circulating microparticles derived from leukocytes, platelets, erythrocytes, endothelial cells, megakaryocytes, and tumors have been identified in numerous studies (Figure 5), their precise role(s) in hemostasis and thrombosis is poorly understood. Scott syndrome patients have a defect that impairs the translocation of phosphatidylserine from the inner to the outer leaflet of the membrane of erythrocytes and platelets. Although the bleeding tendency of these patients has been primarily attributed to decreased platelet procoagulant activity, platelets from Scott syndrome patients also demonstrate defective generation of phosphatidylserine-positive microparticles *in vitro*, suggesting these microparticles are necessary for hemostasis.[88,89] However, it is difficult to separate the role(s) of reduced expression of phosphatidylserine on platelets from reduced production of phosphatidylserine-bearing microparticles. Healthy humans have few circulating leukocyte-derived microparticles; however, the numbers of leukocyte-derived microparticles increase in certain diseases, including cancer[90,91], diabetes[92], and sickle cell disease[93]. Microparticles derived directly from tumors have also been specifically associated with VTE in cancer patients, and are thought to contribute to the thrombotic presentation.[94,95] Expression of procoagulant activity on microparticles (TF and/or phosphatidylserine) has been implicated in microparticle function *in vitro* and *in vivo*.[96–99] One prospective study demonstrated a sharp increase in microparticle TF activity immediately before venous thrombosis in two patients[91], suggesting a causative role of microparticles in the thrombotic event. Murine studies support this observation; human monocyte-derived microparticles promote thrombus formation[100,101] and increase thrombus weight[98] in intravascular thrombosis models. Additional theories regarding the mechanistic role(s) of microparticles in thrombosis invoke their expression of cellular adhesion molecules and ability to activate endothelial cells[102] and leukocytes.

## Contributions of blood flow in thrombosis and bleeding

The effects of blood flow on clotting are perhaps the least interrogated aspect of Virchow's triad. Blood flow in the vascular system is typically characterized by two key variables: shear stress and shear rate. In laminar flow (fluid that flows in concentric, parallel layers), shear stress ($\tau$) is the force per unit area applied between adjacent layers of fluid, and shear rate ($\gamma$, units of inverse time) is the relative velocity gradient between adjacent layers of fluid. For a Newtonian fluid, such as water or plasma, shear stress is the product of fluid viscosity and shear rate. The term "shear stress" when referring to blood flow typically means wall shear stress, which is the tangential force that the fluid imposes directly on the endothelium. For laminar flow in a straight vessel, wall shear stress is directly proportional to the volumetric flow rate (volume per time) and inversely proportional to the third power of the inner vessel diameter. In much of the arterial system, blood vessels adapt their caliber via endothelium-mediated regulation to maintain a mean wall shear stress of approximately 5–20 dynes/cm$^2$.[103,104] This maintenance is thought to be crucial to normal vascular function, including the promotion of antiinflammatory, antithrombotic, anticoagulant, profibrinolytic and antihypertrophic states.[104,105]

Under venous conditions, static or severely reduced flow ("stasis") leads to decreased wall shear stress. Stasis induced by immobility (hospitalization or long-haul air travel) or trauma is associated with venous thrombosis risk, perhaps by modulating the endothelial cell phenotype. The shear stress-responsive Krüppel-like transcription factors (KLF) have been implicated in endothelial cell function.[106–108] Shear stress upregulates KLF expression, inducing expression of antiinflammatory and antithrombotic proteins, including thrombomodulin; whereas, stasis- or proinflammatory cytokine-induced loss of KLF expression leads to enhanced expression of vascular cell adhesion molecule-1 and TF.[109,110]

As such, KLF is an intriguing target for novel antithrombotics aimed at combating stasis-induced thrombosis.

Vascular endothelial cells are also subjected to pressure, a force per area that acts perpendicularly to the vessel wall and modulates endothelial function. Hypertension increases pressure and mechanical damage to arteries, increasing circulating levels of both endothelial- and platelet-derived microparticles.[111] Hypertension may also increase TF exposure *in vivo*; application of pressure to cultured endothelial cells increases TF activity[112] and release of endothelin-1[113].

Flow conditions near a vessel wall are best characterized by wall shear rate. Wall shear rate describes how fast solutes and platelets are delivered within a short, radial distance from the vessel wall, but also the rate at which they are carried downstream as they diffuse away from the wall. Model systems have shown that local flow conditions strongly regulate the delivery of both platelets to a subendothelial surface, and factor X to a wall containing the factor VIIa/TF complex.[114] Typical arterial and venous shear rates are 500–1500 $s^{-1}$ and 10–100 $s^{-1}$, respectively. However, the shear rate and shear stress within a stenotic region significantly increase as the stenosis grows; shear rates may reach 70,000 $s^{-1}$ to 250,000 $s^{-1}$ in severely stenotic vessels.[53,115] Depending on stenosis geometry and location in the vasculature, a recirculation zone (illustrated in Figure 3) may form immediately downstream from the stenotic region. Recirculation zones are often characterized by low shear conditions and long residence times for materials contained within them.[116] Notably, areas of disturbed flow such as branch points and curvatures, especially where wall shear stress is low or oscillatory, correlate with regions of atherosclerotic lesion initiation and stenosis.[104,117]

Shear-dependent expression of platelet P-selectin and formation of monocyte-platelet aggregates suggest that both platelets and leukocytes are activated when they traverse stenotic regions (estimated peak wall shear stress of 2.64 to 281.5 Pa, correlated with stenosis severity), priming these cells for deposition on dysfunctioning or ruptured vasculature and/or contribution to thrombotic events if they become entrapped in the recirculation zone.[116,118] Moreover, soluble agonist-independent accumulation of platelets at a stenotic region is attributed to flow (rheology)-dependent aggregation.[119] As such, flow-dependent changes induced by the stenosis, itself, further increase the risk of thrombosis upon plaque rupture.

The shear rate also dramatically modulates the kinetics of both fibrin monomer formation and polymerization.[120,121] At a constant, given shear rate, initiation of clotting is a function of the size of the exposed region of TF.[120] Once initiated, the shear rate dictates both influx of procoagulant reactants including prothrombin and fibrinogen, and efflux of reaction products including thrombin and fibrin. In *in vitro* assays, increasing shear from 10 to 100 $s^{-1}$ depletes local fibrin monomer concentrations, limiting lateral aggregation and protofibril extension. However, decreasing the shear rate or increasing thrombin generation at a given shear rate increases local monomer concentrations, permitting protofibril and subsequent fibrin fiber formation.[121] Although fibrin networks formed under static (no flow) conditions show an isotropic distribution of fibers with relatively uniform diameters, fibrin produced in flowing blood is oriented along flow vectors[121–123], with a prominent network of thick fibers as well as fiber "aggregates" in which multiple individual fibers are coalesced into bundles.[123] Because the fibrin network's ability to withstand both biochemical dissolution and mechanical disruption is a function of its fiber thickness and branching[20], these studies indicate networks produced under flow would demonstrate significantly different stability than isotropic networks formed under stasis.[123] In fact, using mechanical stress to recapitulate the effects of blood flow on fiber orientation, a recent study found that stretched

fibrin networks are resistant to fibrinolysis[124], underscoring the importance of the effects of blood flow/shear rate on thrombus formation, structure and function.

## Multiple, inter-related "hits" in thrombosis and bleeding

The proposition that multiple "hits" culminate in abnormal coagulation suggests simultaneous consideration of soluble, cellular, and mechanical biomarkers is required to determine clinical risk of thrombosis and bleeding. Indeed, concerted dysfunction in the blood, vessel wall, and blood flow may increase clinical risk via additive or even synergistic mechanisms. Two recent studies have highlighted the need to understand the extent of vessel wall injury when interpreting thrombotic risk related to platelet and plasma protein functions. Hechler *et al*.[125] addressed the controversy regarding the role of GPVI-collagen interactions in thrombosis by showing that thrombi resulting from superficial laser injury depended on platelet GPVI but not procoagulant activity, whereas thrombosis after deeper laser injury that both denuded the endothelium and exposed the adventitia was less dependent on GPVI, but instead could be blocked by hirudin. We have shown that the procoagulant/prothrombotic effects of elevated plasma factor VIII levels can be observed after mild vascular injury (short exposure to $FeCl_3$), but not severe injury to the carotid artery.[126] These studies demonstrate the interdependent relationship between plasma procoagulant activity and vascular function.

Epidemiologic studies in humans support these observations. For example, the prognostic importance of hyperfibrinogenemia appears to be independent of, but additive to, myocardial damage (assessed by troponin-T levels) in patients with unstable coronary artery disease.[35] However, few diagnostic algorithms simultaneously consider markers of vascular damage and plasma hypercoagulability when determining thrombosis risk. Acevedo *et al*.[30] showed that elevated levels of both homocysteine (a trigger of endothelial damage) and fibrinogen contributed to an increased hazard ratio (HR) of 3.29 for 3-year mortality, more than the HR for either homocysteine (HR=2.14) or fibrinogen (HR=2.28), alone. This observation supports the development and use of diagnostic algorithms that incorporate multiple biomarkers that indicate not only blood composition, but also vascular function, when determining clinical interventions and duration of anticoagulant therapy.

Clearly then, delineating the operant pathophysiologic mechanisms necessitates understanding the substantial cross-talk and feedback pathways that connect the aspects of Virchow's triad. However, a significant impediment to these investigations is the difficulty in simulating and collecting data on multiple biochemical, cellular and mechanical processes simultaneously. Mathematical and computational modeling is being used to circumvent this limitation. Indeed, models of coagulation kinetics have been instrumental in delineating TF regulatory mechanisms[127,128], sensitivity of this pathway to initial TF concentration[129], and threshold behavior of thrombin production and the importance of binding site density on the reactive surface of platelets.[130,131] Fewer models have incorporated the effects of flow in coagulation, and only a subset have generated hypotheses that were subsequently tested and validated experimentally[132–134]. Of note, theoretical work by Kuharsky and Fogelson[130] led to the hypothesis that platelets physically inhibit subendothelial activity. This hypothesis was later given experimental support[135] and incorporated into the biological literature[136,137].

Two models aimed to capture interplay between the biochemistry, blood cells and fluid environment and predict clot behavior in multiple parameter regimes. Xu *et al*.[138] developed a computational model that invoked the cellular potts model to represent platelets in flow, which allowed for tracking of individual discrete platelet behaviors. Coagulation biochemistry was based on the system described by Jones and Mann[128] and did not include

surface-dependent reactions. The model predicted that red blood cell entrapment within the growing platelet thrombus led to thrombus heterogeneity and structural instability. Leiderman and Fogelson[139] developed a model that included detailed biochemistry, including surface-dependent reactions, chemical activation and deposition of platelets. The model tracked platelet concentrations rather than discrete objects and treated the growing platelet mass as a porous material. This feature allowed analysis of fluid and solute transport within the growing mass, and showed that diffusive transport within the mass promotes its upstream growth. Furthermore, this model showed thrombus growth was strongly influenced by wall shear rate and the near-wall excess of platelets. Although both models substantially advance simulation of the complex and coupled blood clotting system under flow, the predictions made by these models remain to be tested in biological assays. Moreover, all of these models still lack features known to affect coagulation including effects of platelet-platelet cohesion and clot dissolution. However, improved computing power, continued refinement of computational models and incorporation of new experimental data into the models are expected to identify novel therapeutic targets for the treatment of thrombosis and bleeding.

## Conclusions

Understanding the interplay between the components of Virchow's triad is necessary to effectively diagnose and treat bleeding and thrombotic disorders. Increasing awareness of the complexities of these presentations, along with increasingly sophisticated technologies to analyze soluble, cellular, and physical dysfunctions in concert will shed new light on these pathologies and identify novel therapeutic targets. With such targets, we may achieve the long-sought, but thus far elusive, goal for coagulation treatment: safe and effective antithrombotic and hemostatic approaches that are not complicated by bleeding or thrombotic risks.

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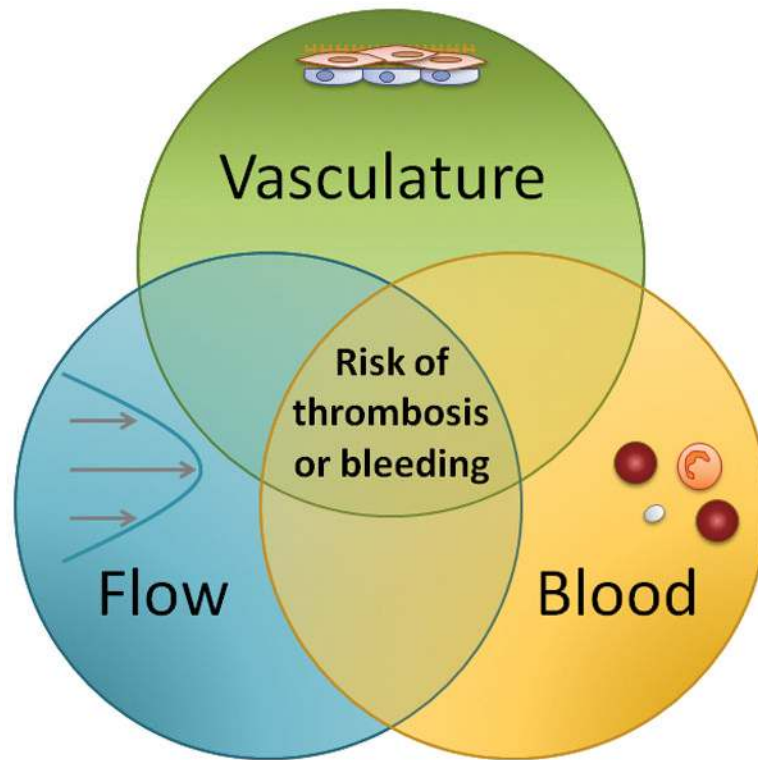
**Figure 1.**
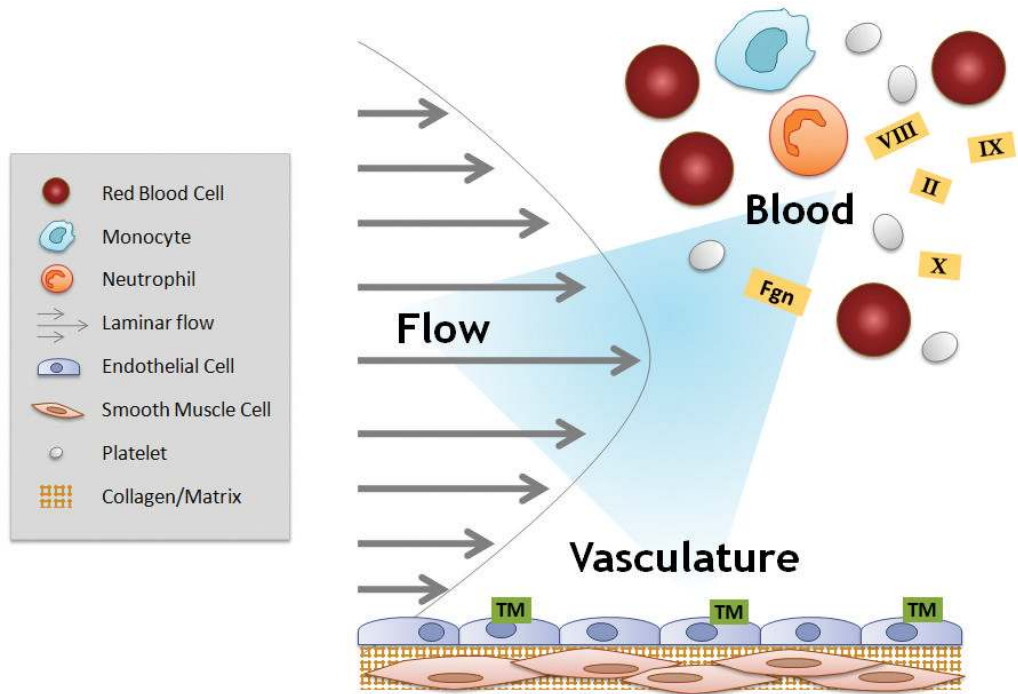Venn diagram illustrating the propensity of thrombosis and bleeding at the intersection of abnormalities in blood composition, vessel wall function, and blood flow/shear.

**Figure 2. Schematic showing the elements of Virchow's triad**
This conceptual model describes the three components (blood flow, blood composition, vascular function) that regulate coagulation. Abbreviations: IX, factor IX; VIII, factor VIII; II, prothrombin; Fgn, fibrinogen; TM, thrombomodulin
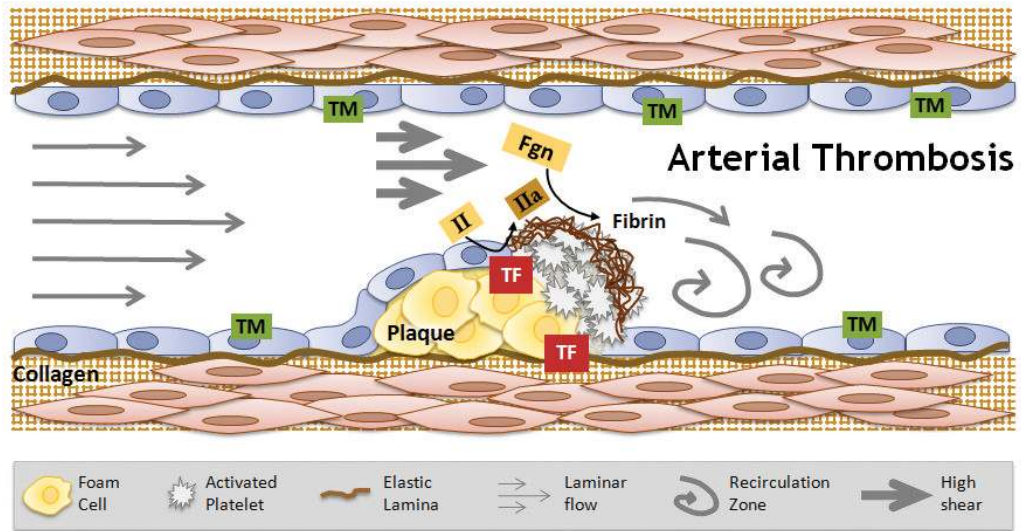
**Figure 3. Interplay between abnormalities in blood components, the vasculature, and blood flow contribute to the development of arterial thrombosis**

Arterial thrombosis involves the formation of platelet-rich "white clots" that form after rupture of atherosclerotic plaques and exposure of procoagulant material such as lipid-rich macrophages (foam cells), collagen, tissue factor and/or endothelial breach, in a high shear environment. Abbreviations: TM, thrombomodulin; II, prothrombin; IIa, thrombin; Fgn, fibrinogen; TF, tissue factor
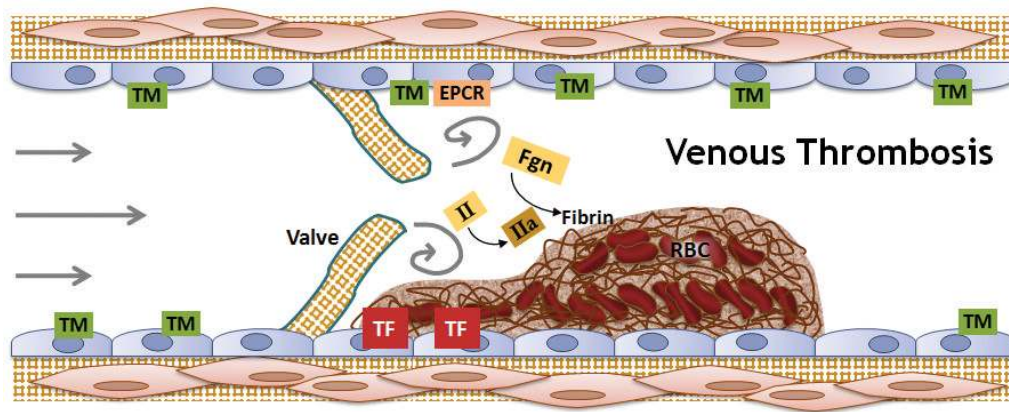
**Figure 4. Interplay between abnormalities in blood components, the vasculature, and blood flow contribute to the development of venous thrombosis**
Venous thrombosis involves the formation of fibrin-rich "red clots" that result from exposure of procoagulant activity on intact endothelium plus plasma hypercoagulability, in reduced or static blood flow. Venous thrombi are thought to initiate behind valve pockets, where reduced or static flow decreases wall shear stress that normally regulates endothelial cell phenotype. Abbreviations: TM, thrombomodulin; EPCR, endothelial protein C receptor; II, prothrombin; IIa, thrombin; TF, tissue factor; Fgn, fibrinogen; RBC, red blood cells
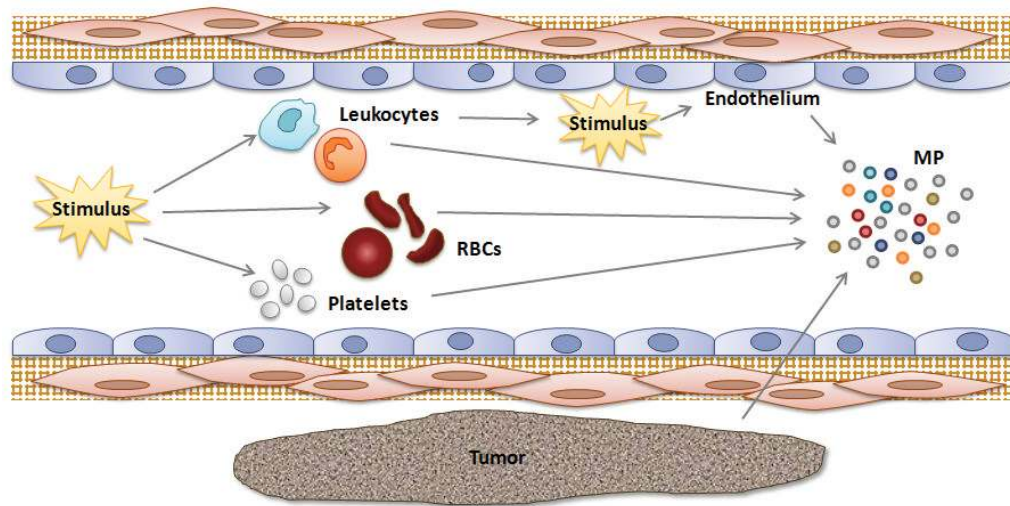
**Figure 5.**
Circulating microparticles are derived from a variety of cell types including leukocytes, platelets, megakaryocytes, red blood cells, endothelial cells, and tumors. Microparticles (MP) carry cell-specific markers and functional properties of their parent cell.