Pathogenesis of thrombosis: cellular and pharmacogenetic contributions

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Abstract: Our understanding of thrombosis formation has evolved significantly ever since physician Rudolf Virchow proposed his "triad" theory in 1856. Modern science has elucidated the mechanisms of stasis, hypercoagulability, and endothelial dysfunction. Today, we have a firm understanding of the key molecular factors involved in the coagulation cascade and fibrinolytic system, as well as the underlying genetic influences. This knowledge of cellular and genetic contributors has been translated into diverse pharmaceutical interventions. Here, we examine the molecular and cellular mechanisms of thrombosis and its associated pathologies. We also review the current state of pharmacologic interventions, including pro- and anti-thrombotics, direct oral anticoagulants, and anti-platelet therapies. The pharmacogenetic factors that guide clinical decision making and prognosis are described in detail. Finally, we explore new approaches to thrombosis drug discovery, repurposing, and diagnostics. We argue that network biology tools will enable a systems pharmacology revolution in the next generation of interventions, facilitating precision medicine applications and ultimately leading to improved patient outcomes.

Keywords: Anticoagulants; blood coagulation; thrombosis; precision medicine; pharmacogenetics

Introduction

Thrombosis is defined as the formation of a blood clot (thrombus) within a blood vessel (1). This process is generally considered pathologic except in the case of traumatic injury, where thrombus formation may prevent loss of blood volume and protect against systemic infection. A rudimentary description of the factors contributing to thrombosis is attributed to Rudolf Virchow. A modern interpretation of this "Virchow's triad" is that hemodynamic disruption, intrinsic hypercoagulability, and endothelial damage or dysfunction are the three broad categories that lead to thrombosis (*Figure 1*) (2).

When the balance of the body's pro- and anti-thrombotic forces becomes disturbed, it is often necessary to treat pharmacologically in order to restore homeostasis. In the hemophiliac, this means administration of pro-thrombotics such as recombinant activated factor VII (rFVIIa), while in the patient with atrial fibrillation, an anti-thrombotic such as warfarin is required. These agents may act directly or indirectly on the coagulation cascade, platelets, or other components to achieve their effect. That effect, like in so much of pharmacology, may be influenced by the individual's genetic make-up, and in some cases those genetics may seriously alter the drug's effect. Here, we will discuss drugs with pro- and anti-thrombotic effects, their mechanisms of action, and the influence of genetic polymorphisms on their effect.
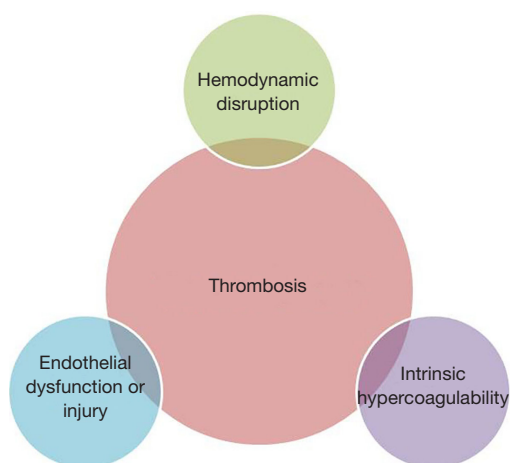
Figure 1 Virchow's triad, a modern interpretation.

Pro- and anti-thrombotic pharmacology and genetic influences

Pro-thrombotics

Drugs that intentionally encourage coagulation are often concentrates of coagulation cascade proteins, such as rFVIIa, which contains activated factor VII; prothrombin complex concentrates (PCC), which contain factors II, IX, X and sometimes VII; and fresh frozen plasma (FFP), which contains all the coagulation factors (3). These may be administered to patients with a coagulation disorder or to patients suffering from massive bleeding due to trauma or an overdose of an anti-thrombotic. By their nature, these drugs increase the risk of thrombosis, but genetic variation in drug response has not been explored.

More common are the drugs that unintentionally encourage coagulation, which is often an undesirable side effect of the drug (*Table 1*). Such drugs are numerous, but classic examples are oral contraceptives (OCPs) and chemotherapy agents.

Oral contraceptives containing an estrogen and a progestogen increase the risk of venous thromboembolism (VTE) via increased levels of thrombin and fibrin, as well as the levels of almost all coagulation factors (4,5). Plasma from patients taking OCPs is also resistant to the anticoagulation effect of activated protein C, an independent risk factor for VTE (4). OCPs also increase the levels of factor VII-activating protease (FSAP), which activates factor VII, thus initiating the extrinsic path of the coagulation cascade (6). In OCP-taking individuals with the 1601GG genotype of

the *FSAP* gene, FSAP levels and consequent factor VII activation were higher than in the 1601GA genotype (6). Genetic variation in the metabolism of a component of combined oral contraceptives, ethinylestradiol, has been linked with increased VTE risk. Possession of the A or B *CYP3A4* haplotype or having two copies of the D *UGT2B7* haplotype increased VTE risk in patients on OCPs but not in those not taking OCPs (5). The *UGT2B7* haplotype D also increased the levels of sex-hormone-binding-globulin, a marker of VTE risk. Another study of OCPs found that possession of the A allele of the *F11 rs2289252* polymorphism—a variant in factor XI associated with VTE in the general population—increased VTE risk (7).

Thalidomide has returned to the marketplace, this time for treatment of multiple myeloma. Though its precise mechanism of action remains unclear, it appears to exert its effect both on the malignant cell as well as the microenvironment (8). Unfortunately, it increases the risk of VTE, perhaps due to effects on COX-2 or cytokines. SNPs that may affect risk of VTE in thalidomide-treated patients include those involved in response to DNA damage, cytokine balance, and several genes involved in apoptosis (8). Polymorphisms in the coagulation cascade did not appear to be associated with thalidomide-associated VTE.

Bevacizumab is a recombinant humanized monoclonal neutralizing antibody targeting vascular endothelial growth factor (VEGF) and is used in a variety of malignancies (9). It has also been associated with an increased risk of thromboembolic events (9,10). Among glioma patients, those treated with bevacizumab had increased incidence of ischemic stroke, but the same rates of deep vein thrombosis (DVT) (10). Possession of the CC genotype of the *rs2010963* SNP on the vascular endothelial growth factor A (VEGFA) gene in glioma patients was associated with an increase in thrombo-hemorrhagic events during treatment with bevacizumab (11). In a xenograft mouse model of human lung tumors, bevacizumab appeared to exert its pro-thrombotic effect through blocking VEGF's inhibition of plasminogen activator inhibitor (PAI-1), thus increasing PAI-1 expression and increasing risk of thrombosis (9).

Anti-thrombotics

Pharmacologic anticoagulation is more commonly seen than pro-coagulation (*Table 2*), and foremost among the anticoagulants is warfarin. Despite increasing prevalence of

Table 1 Pro-thrombotic agents and relevant genetic polymorphisms

Drug	Gene polymorphism	Protein role	Effect
OCPs	*FSAP 1601GG*	Activates factor VII	Increased factor VII activation
	CYP3A4 haplotype A or B	Metabolizes ethinylestradiol	Increased VTE risk
	UGT2B7 haplotype D	Metabolizes ethinylestradiol	Increased sex-hormone-binding-globulin, increased VTE risk
	F11 rs2289252-A	Factor XI	Increased VTE risk
Bevacizumab	VEGFA *rs2010963*-CC	Mediates angiogenesis	Increased thrombo-hemorrhagic events in glioma patients

OCPs, oral contraceptives; VTE, venous thromboembolism.

Table 2 Anti-thrombotic agents and relevant genetic polymorphisms

Drug	Gene polymorphism	Protein role	Effect
Warfarin	*VKORC1*2, *3, *4*	Reduces vitamin K epoxide	Increased warfarin effect
	*CYP2C9*2, *3*	Metabolizes S-warfarin	Increased warfarin effect
Dabigatran	*ABCB1 rs4148738*	Transports dabigatran	Increased dabigatran plasma levels
	CES1 rs2244613	Metabolizes dabigatran etexilate	Decreased trough dabigatran levels, reduced risk of bleeding
	CES1 rs8192935-T	Metabolizes dabigatran etexilate	Decreased trough dabigatran levels
Rivaroxaban	*ABCG2* Q14K	Transports rivaroxaban	Unknown
Heparin	*FcγRIIA*-H131R	Mediates antibody-platelet binding	Increased HIT-related thrombosis risk
	GpIIb/IIIa-HP-1	Platelet receptor for fibrinogen	Increased HIT-related thrombosis risk
	PECAM1-L125V	Platelet adhesion protein	Increased HIT-related thrombosis risk
	CD148 276P and 326Q	Downstream in FcγRIIA signaling pathway	Decreased risk of HIT
Aspirin	*PTSG2 rs20417*-C	Encodes COX-2	Decreased risk of major cardiovascular events
	PEAR1 rs12041331-AA	Mediates platelet-platelet aggregation	Reduced platelet aggregation
	ITGB3 P1A1/A2	Platelet receptor for fibrinogen	Reduced aspirin efficacy in healthy subjects
	CYP4F11-GG	Metabolizes aspirin	Increased small bowel bleeding
	CYP2D6-GG	Metabolizes aspirin	Increased small bowel bleeding
	CYP24A1-T	Metabolizes aspirin	Increased small bowel bleeding
	GSTP1-G	Metabolizes aspirin	Increased small bowel bleeding
Clopidogrel	*CYP2C19*2, *3*	Metabolizes clopidogrel	Decreased clopidogrel effect
	*CYP2C19*17*	Metabolizes clopidogrel	Increased clopidogrel effect
Ticagrelor	*CYP4F2 rs3093235*-TT	Metabolizes ticagrelor	Increased anti-platelet effect, increased risk of bleeding

the direct oral anticoagulants (DOACs), warfarin remains the most commonly prescribed anticoagulant. A classic example of pharmacogenetics is warfarin and its interactions with two genes: cytochrome P450 2C9 (*CYP2C9*) and vitamin K epoxide reductase (*VKORC1*) (12). Single nucleotide polymorphisms (SNPs) in these genes alter the effectiveness of warfarin. Warfarin acts by inhibiting VKORC1, which is responsible for the reduction of vitamin K (13). Without the reductase, vitamin K is unable to be utilized by the vitamin K-dependent clotting factors: factors II, VII, IX, and X. Warfarin thus indirectly inhibits the clotting cascade via its inhibition of the reductase, resulting in anticoagulation.

Warfarin is notorious for its dose variability, and depending on the patient population, genetic factors may account for up to 45% of this variability (14). There are two key genetic polymorphisms that influence warfarin's effect. The first are polymorphisms in a gene encoding a subunit of vitamin K epoxide reductase, *VKORC1*. The second are polymorphisms in the cytochrome P450 (CYP) responsible for the metabolism of warfarin, CYP2C9. Polymorphisms in both these structures result in increased sensitivity to warfarin, and hence increased risk for severe bleeding (15). The *VKORC1*2* allele is a marker of warfarin sensitivity in 93% of patients (16). Like other alleles *3 and *4, *VKORC1*2* results in decreased VKORC1 expression, lower protein production, and thus an inflated effect of warfarin. Two common *CYP2C9* variants, *2 and *3, are both the result of single amino acid substitutions and reduce the metabolism of the more potent enantiomer S-warfarin by 30–50% (*2) and 90% (*3), and thus increase the duration of warfarin's effect (13,16). Those patients with *VKORC1* and *CYP2C9* polymorphisms that increased their sensitive to warfarin were shown to benefit more from edoxaban treatment as compared with warfarin than normal responders (17).

Direct oral anticoagulants

The DOACs are relatively new anticoagulants that act either by directly inhibiting thrombin in the case of dabigatran, or by directly inhibiting activated factor X, as in the case of rivaroxaban, apixaban, and edoxaban (15). Due to their recent introduction, there is less data on the influence of genetics on DOAC efficacy, but genetic variability does seem to play a role. All DOACs are substrates of the transport protein P-glycoprotein (P-gp),

encoded by *ABCB1* (18). Variations in P-gp expression have been seen in individuals with SNPs in exon 21 and exon 26 of *ABCB1*. Possession of the *ABCB1 rs4148738* polymorphism (in linkage disequilibrium with *rs1045642* on exon 26) resulted in higher peak dabigatran plasma concentrations than those without the polymorphism (18). Rivaroxaban requires both P-gp and breast cancer resistance protein (BCRP, encoded by *ABCG2*) for absorption and excretion (17). A polymorphism of *ABCG2*, Q141K, reduces activity of BCRP, resulting in reduced transport of its substrates, though this has not yet been studied in the case of rivaroxaban (17). However, in mouse models, lack of P-gp and BCRP greatly reduced clearance of rivaroxaban.

Dabigatran etexilate, a pro-drug, is converted to its active metabolite by esterases, including CES1. CYPs do not play a role. Individuals with the *CES1* polymorphism *rs2244613* had 15% lower trough dabigatran concentrations and reduced risk of bleeding (15,19). Possession of the T variant of the *CES1* SNP *rs8192935* also lowered trough dabigatran concentrations (20).

Less than 4% of edoxaban is metabolized by cytochrome enzymes, primarily hydrolysis via CES1 (17). Given that polymorphisms in *CES1* affect dabigatran serum levels, these polymorphisms may also affect edoxaban, though such studies have not been conducted, and edoxaban does not require bioactivation.

Unlike dabigatran and edoxaban, rivaroxaban and apixaban are heavily metabolized by CYPs, and like many drugs, CYP3A4 plays a large role (14). Two-thirds of rivaroxaban is metabolized by CYPs, most prominently CYP3A4 and CYP2J2 (17). Although none of the 44 polymorphisms of *CYP3A4* or the 10 polymorphisms of *CYP2J2* have been studied in relation to rivaroxaban, these polymorphisms have been associated with variability in enzyme activity level, suggesting possible effect on rivaroxaban metabolism (17).

Like rivaroxaban, apixaban is also metabolized by CYP3A4, and CYP3A5 plays an important role too, as well as CYP1A2 and CYP2J2 to lesser extents (17). The conjugation of the inactive apixaban metabolite, O-demethyl apixaban, to another inactive product is performed by SUT1A1, of which three major genotypes with differing enzymatic activity exist (17). Again, no studies exist as to the possible influence of these polymorphisms on apixaban, but reduced activity of a downstream reaction has the potential to influence the metabolism of apixaban.

Heparin

The mainstay of inpatient anticoagulation is heparin, and few complications are as feared as heparin-induced thrombocytopenia (HIT). This mass activation of platelets and consequent widespread thrombosis is due to an immune response involving IgG antibodies targeted against complexes of platelet factor 4 and heparin, and can occur in patients receiving unfractionated or low molecular weight heparin (21,22). HIT has a mortality of greater than 30% and occurs in 0.5–5% of patients treated with heparin (21). Using genetic variability to predict patients likely to develop HIT is thus of great interest. Some promising genes include SNPs at *HLA-DRA*, an area already associated with several immune-mediated adverse drug reactions, and *TDAG8*, which plays a role in activation-induced apoptosis of T cells and attenuation of immune-mediated cytokine production (21). Alleles associated with an increased risk of thrombosis in HIT include the polymorphisms *FcγRIIA*-H131R, encoding an antibody binding protein; *GpIIb/IIIa*-HP-1, which encodes the platelet receptor for fibrinogen; and *PECAM1*-L125V, encoding a platelet adhesion protein (23). The development of thrombosis in individuals with HIT and the *FcγRIIA*-H131R polymorphism may be due to more efficient activation of platelets in homozygous RR individuals (22). Particularly key for the development of HIT is the FcγRIIA receptor, the only Fc receptor on platelets, which mediates the interaction between platelets and antibodies and when stimulated activates platelets (22). A membrane protein tyrosine phosphatase involved in downstream signaling of the FcγRIIA receptor, *CD148* polymorphisms 276P and 326Q were associated with decreased risk of HIT, suggesting a less efficient FcγRIIA signaling pathway in individuals with these alleles (22).

Anti-platelet therapy

Another standard of anticoagulation is anti-platelet therapy, most commonly represented by aspirin and clopidogrel. One of the most popular drugs in the world, aspirin renders platelets non-functional by irreversibly inhibiting platelet cyclooxygenase (primarily COX-1), responsible for the ultimate production of thromboxane A2, an activator of the coagulation cascade (17). Despite exerting its primary effect on COX-1, aspirin also affects COX-2, and variations in the *PTSG2* gene that encodes COX-2 may play a role in variability of response to aspirin. Possession of the *rs20417*

C allele of *PTSG2* conferred a decreased risk of major cardiovascular events, which was particularly significant in aspirin users (17). Another clinically significant polymorphism is that on the platelet endothelial aggregation receptor 1 gene, *PEAR1*, important for signaling in platelet-platelet aggregation. Reduced aggregation in response to both collagen and epinephrine was observed in aspirin-taking patients with the *rs12041331* minor AA allele, present in 8–28% of populations, as compared with GG or GA genotypes (17). Finally, possession of the P1A1/A2 SNP on the *ITGB3* gene, which encodes the IIIa subunit of the GPIIb/IIIa receptor, has been linked with reduced efficacy of aspirin in healthy subjects, but not in those with cardiovascular disease (17).

A side effect of aspirin therapy is increased bleeding risk, particularly gastrointestinal. Small bowel bleeding associated with low dose aspirin therapy was found to be more common patients with the GG homo-genotypes of *CYP4F11* or *CYP2D6*, those with the T allele of *CYP24A1*, or the G allele of *GSTP1* (24).

Clopidogrel irreversibly inhibits the P2Y12 purinergic receptor on platelets, preventing stimulation by adenosine diphosphate, thus inhibiting platelet aggregation (25). Clopidogrel is a pro-drug, of which approximately 15% is metabolized by a variety of CYPs in a two-step process to form the active metabolite. CYP2C19 plays key roles in both steps, while CYP3A4 is essential for the second step. Polymorphisms in the genes encoding these enzymes may result in altered clopidogrel efficacy. *CYP2C19* has two reduced function alleles—*CYP2C19*2* and *3*—formed via single nucleotide polymorphisms of guanine to adenine (25). The *17 variant is associated with increased gene transcription and thus increased enzyme activity. Clinically, presence of a *2 or *3 allele results in reduced metabolism of CYP2C19 drugs, including clopidogrel, while presence of a *17 allele results in enhanced metabolism. For a pro-drug such as clopidogrel, this means that individuals with a *2 or *3 allele will have lower plasma concentrations of the active metabolite, and hence require a higher dose for therapeutic effect, while those with a *17 allele will have higher plasma concentrations of the active metabolite, and require a lower dose of clopidogrel. Polymorphisms in a variety of other enzymes have been suggested to influence clopidogrel efficacy, but the *CYP2C19* variants remain the most clinically relevant at the present time (25).

Alternatives to clopidogrel include ticagrelor, which binds reversibly to P2Y12 receptor on platelets, and is not a

pro-drug, hence requiring no activation by hepatic enzymes. Like clopidogrel, however, ticagrelor also appears to have some genetic influencers, such as the *CYP4F2 rs3093235* TT variant, which was associated with an increased anti-platelet effect and increased risk of bleeding (26).

Systems approaches to elucidating pharmacogenetics of thrombosis

We have discussed the current state of pharmacogenetics for pro- and anti-thrombotic agents. This field has become an indispensable part of the clinical toolkit in managing patients with coagulation disorders. The DNA sequence alone, however, does not explain the entire story and consequently does not fully predict patients' responses. This is especially true for underrepresented populations in clinical trials, comorbid conditions, and polypharmacy (27). In other words, for the vast majority of drugs and patients, we receive little guidance from pharmacogenetics today.

To improve the state of the practice and move toward a precision medicine ideal, we must move beyond biological reductionism and pursue a systems approach—one where physiological networks of biomolecules are taken into account. Computational methods are now revealing the deep functional organization and emergent behavior of human biology (28,29). These advances are also opening doors to applying systems and network biology tools to pharmacology, providing novel insights into drug purposing and safety (30-32).

Some early work in this field has been applied to the pathogenesis of thrombosis. A topological analysis of platelet signaling, coagulation, and resolution has shed some light on the complex mechanisms of some traditional Chinese medicines (33). Comprehensive functional enrichment and pathway analysis has elucidated a link between thrombosis and myeloproliferative disorders, reinforcing Virchow's triad model and indicating that some drugs approved for myeloproliferative disorders could be repurposed to treat thrombosis (34). Protein-protein interaction (PPI) network analysis followed by pathway enrichment analysis identified interleukin-6 and other immunoregulatory proteins as having a key role in inflammation-driven atherosclerosis (35). This approach could suggest potential drug targets for alleviating thrombosis due to endothelial dysfunction.

Going forward, we suggest expanding investigation of published datasets using newer network biology techniques. Tools like NetDecoder and P-Map could facilitate the

development of new molecular entities, combination therapies, and repurposing of existing pharmaceuticals (36,37). Context-specific, prioritized biological networks and gene activities, as well as phenotype mapping, are essential for understanding the disease process deeper than Virchow ever imagined. We envision that these systems pharmacology methods could complement or even supplant current pharmacogenetics strategies to serve as complementary prognostics and diagnostics for therapeutic decision making.

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Footnote

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