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Abstract

People with haemophilia (PWH) have an increased tendency to bleed, often into their joints, causing debilitating joint disease if left untreated. To reduce the incidence of bleeding events, PWH receive prophylactic replacement therapy with recombinant factor VIII (FVIII) or factor IX (FIX). Bleeding events in PWH are typically proportional to their plasma factor VIII or IX levels; however, in many PWH, bleeding tendency and the likelihood of developing arthropathy often varies independently of endogenous factor levels. Consequently, many PWH suffer repeated bleeding events before correct dosing of replacement factor can be established. Diagnostic approaches to define an individual's bleeding tendency remain limited. Multiple modulators of bleeding phenotype in PWH have been proposed, including the type of disease-causing variant, age of onset of bleeding episodes, plasma modifiers of blood coagulation or clot fibrinolysis pathway activity, inter-individual differences in platelet reactivity and endothelial anticoagulant activity. In this review, we summarise current knowledge of established factors modulating bleeding tendency and discuss emerging concepts of additional biological elements that may contribute to variable bleeding tendency in PWH. Finally, we consider how variance in responses to new gene therapies may also necessitate consideration of patient-specific tailoring of treatment. Cumulatively, these studies highlight the need to re-consider the current 'one size fits all' approach to treatment regimens for PWH and consider therapies guided by the bleeding phenotype of each individual PWH at the onset of therapy. Further characterisation of the biological bases of bleeding heterogeneity in PWH, combined with the development of novel diagnostic assays to identify those factors that modulate bleeding risk in PWH, will be required to meet these aspirations.

Keywords: Haemophilia, bleeding heterogeneity, coagulation, fibrinolysis, gene therapy

Introduction

The biochemical and cellular response to vessel injury results in the formation of a haemostatic plug that prevents excessive blood loss[1]. This plug consists of platelets which are recruited to the site of injury. Here, they adhere to the newly exposed sub-endothelial surface and become activated, undergoing morphological changes that allow platelet aggregation[1-3]. Concurrently, endothelial disruption prompts tissue factor (TF) exposure and binding to activated factor VII (FVIIa) in the blood. This complex triggers a series of protease activation events that culminate in the generation of thrombin, which catalyses fibrin deposition around the platelet plug. Thrombin also catalyses FXIII activation and activated FXIII (FXIIIa) increases clot stability by cross-linking fibrin[4,5]. Clot growth is controlled by the actions of endogenous anticoagulants, including antithrombin (AT), which inhibits coagulation proteases, tissue factor pathway inhibitor (TFPI), which attenuates TF-driven initiation of blood coagulation and the protein C pathway, which dynamically controls the generation of thrombin[4,6].

Activation of the intrinsic pathway by thrombin is critical for the generation of sufficient thrombin for fibrin deposition. Factor VIII (FVIII) and factor IX (FIX) are activated as part of this pathway and form the the intrinsic tenase complex with factor X[4,5]. Haemophilia A (HA) is an X-linked bleeding disorder caused by mutations in the *F8* gene that cause reduction of plasma FVIII levels and/or activity. Similarly, haemophilia B (HB) is caused by mutations in the *F9* gene that result in plasma FIX deficiency. Plasma from individuals with either HA or HB exhibit impaired intrinsic tenase complex activity, slowing thrombin generation and diminishing thrombin generation[7]. Individuals with either HA and HB have an elevated risk of bleeding, either spontaneously or following injury. This most commonly manifests as bleeding into 'target' joints, leading to chronic synovitis and degenerative arthritis if left untreated. People with haemophilia (PWH) are categorised into '*mild*' (5-40% of normal FVIII levels), '*moderate*' (1-<5% of normal FVIII levels) or '*severe*' (<1% of normal FVIII levels) groups, based solely on their residual FVIII or FIX plasma levels. For individuals with severe haemophilia, prophylactic replacement of missing factor to raise plasma factor levels to those of 'moderate' HA or HB individuals has become the current standard of care for PWH. This approach is based on the premise that factor levels are directly proportional to bleeding tendency; however, it is now well-established that factor levels do not always match the clinical bleeding phenotype of PWH[8]. Despite the success of prophylactic factor replacement, ~10% of PWH exhibit sustained bleeding which does not correlate with their factor levels[9,10]. Similarly, factor levels do not predict the frequency or severity of bleeding events in people with severe

haemophilia (PWSH) who have a milder bleeding tendency and who do not require regular prophylactic factor infusion[10,11].

These studies demonstrate that other biological elements beyond FVIII or FIX levels significantly influence bleeding phenotype in PWH. Nevertheless, the underlying cause for this phenotypic heterogeneity is still poorly understood. In this review, we outline current knowledge on genetic, biochemical, cellular and clinical mechanisms that may influence bleeding phenotype independent of FVIII or FIX levels. Many potential mechanisms have been advanced to explain the underlying basis of bleeding heterogeneity in PWSH (Figure 1). In particular, the nature of the genetic disease causing variants that cause haemophilia can impact on phenotype[12,13] and co-inheritance of prothrombotic polymorphisms can result in a rebalancing of haemostasis that may result in a milder bleeding tendency[14,15]. Additionally, differences in individual procoagulant, anti-coagulant and fibrinolytic pathway activities have also been postulated to influence bleeding rates[11,16-18] (Figure 2).

Age at first joint bleed as an indicator of subsequent bleeding tendency

Despite having similar FVIII/FIX levels (<1%), there is significant variability in bleeding pattern within the PWSH population. In general, PWSH experience their first joint bleed at an earlier age than mild or moderate PWH[19,20]. However, age of first joint bleed in PWSH ranges from 3 months up to 6 years[20,21]. Previous studies have shown an inverse correlation between age of onset of joint bleeding and bleeding severity, in that PWSH who experience their first joint bleed later in life experience fewer subsequent bleeds and have a reduced risk of orthopaedic complications[21,22]. Consequently, those individuals with later recorded bleeds have significantly lower prophylactic treatment requirements than those PWSH with bleeding events detected early in life[21] and may possess additional biological determinants that mitigate their bleeding tendency. The point at which PWH begin prophylaxis can also impact on the severity of bleeding events and development of arthropathy later in life. PWH who receive a prophylactic treatment plan before or directly following their first joint bleed are significantly less likely to suffer from arthropathic complications[19,23]. In contrast, those PWH who began prophylactic treatment following several prior joint bleeds are much more likely to develop serious joint damage[23-25]. These studies emphasise the importance of early initiation of prophylactic treatment in PWSH to preserve joint health and minimise the number of annual bleeds experienced.

Differences in bleeding phenotype between people with HA and HB

People with severe HB often present with a less intense bleeding phenotype than their HA counterparts and are reported to have a lower number of annual bleeds, less recombinant factor consumption and reduced likelihood of haemophilic arthropathy[26,27]. The molecular basis for this anomaly is unclear. One potential explanation may relate to differences in physiological processing and storage of FVIII and FIX. While FVIII is largely found only in the intravascular space after secretion, FIX is also stored in extravascular tissue. Currently, the mechanism(s) underlying FIX migration outside the vascaulature in the absence of injury remain to be determined, although FIX binding to collagen IV appears critical for extravascular storage. This is evidenced by the higher plasma levels and reduced extravascular FIX after administration of recombinant FIX variants with reduced collagen affinity to *F9*-deficient mice. Extravascular compartmentalisation via collagen IV binding may significantly modulate FIX pharmacodynamics, resulting in prolonged FIX bioavailability compared to the exclusively intravascular FVIII[28-30]. These studies also suggest that measurement of plasma FIX levels in a laboratory setting account for an unknown percentage of available FIX and that uncharacterised tissue FIX levels may vary significantly between individual PWH. Pertinently, it has been shown that total FIX levels may be three-fold higher than that which can be measured in the plasma[29]. These differences in FVIII and FIX storage and processing may also contribute to the divergent rates of musculoskeletal complications between people with HA and HB. People with HA, in particular, are significantly more likely to undergo orthopaedic surgery as a result of haemophilic arthropathy compared to people with HB[31]. Interestingly, people with HA have higher ultrasound scores indicative of joint damage and significantly reduced levels of osteoprotegerin in their synovial fluid, a protective agent in haemophilic arthropathy[32]. Cumulatively, these studies demonstrate that people with severe HB are less likely to suffer from a severe bleeding tendency that HA, although the biology underpinning these clinical differences are still not fully understood.

Inter-individual genetic differences that modify bleeding tendency in PWH

The presence of null or non-null *F8* or *F9* mutations has been postulated to impact bleeding phenotype. Null mutations prevent protein synthesis, whereas non-null *F8* mutations allow for the synthesis of low, but detectable factor levels. ~50% of severe HA PWH have inversions on intron 22 of the *F8* gene [33,34]. PWSH with *F8* non-null mutations have been reported to exhibit a milder bleeding phenotype compared to PWH with *F8* null mutations[11,13]. In contrast, non-null missense mutations are more prevalent in individuals with HB[35,36], which may

contribute to the observed disparity between clinical presentation in people with severe HA and HB[26,37,38].

Other proposed modulators of bleeding tendency in PWH include co-inheritance of common prothrombotic gene variants, in particular factor V Leiden (FVL). FVL causes FVa proteolysis by activated protein C (APC) to be partially inhibited, causing impaired restriction of prothrombinase activity[14,39]. FVL heterozygosity occurs in ~5% of Caucasian populations and Individuals heterozygous for FVL have a 7-fold increased risk of venous thromboembolism, whereas individuals with homozygous FVL possess an ~25-fold increased VTE risk. Interestingly, PWH who co-inherit FVL heterozygosity exhibit a less intense bleeding phenotype than PWH without FVL, highlighting its potential role in re-balancing haemostasis in PWH[14,40]. This is supported by animal studies in which HA and HB mice with FVL exhibit less severe bleeding than those haemophilic mice without FVL[41]. Specifically, HA and HB mice that were either heterozygous or homozygous for FVL showed significantly reduced activated partial thromboplastin time (aPTT) and a corresponding increase in thrombin-antithrombin (TAT) levels. Following endothelial laser injury, these mice also exhibited enhanced local clot formation. Notably, co-inheritance of the prothrombin G20210A mutation, which results in increased plasma prothrombin levels, has also been reported to mediate a similar protective effect as FVL upon bleeding tendency in PWH[8]. Similarly, protein C deficiency in PWH leading to reduced bleeding phenotype has been reported, with correspondingly enhanced thrombin generation compared to other PWH[42]. Some studies have observed lower plasma levels of TFPI in severe HB compared to HA, suggesting it as another potential mechanism to explain the often less severe bleeding phenotype in individuals with severe HB compared to HA[43]. Furthermore, a potentially protective role of reduced plasma TFPI levels was also studied using a murine HA model, where co-deletion of TFPI and FVIII resulted in increased clot volume and reduced bleeding[44].

Although these ‘re-balancing’ prothrombotic mutations alone cannot account for phenotypic heterogeneity in all PWH, these studies underscore the potential value of efforts to utilise non-factor therapies to re-balance haemostasis in PWH, in particular non-factor haemostatic agents that restore thrombin generation by inhibition of endogenous anticoagulant pathways[45-47]. The observation that diminished TFPI activity can restore haemostasis in haemophilia prompted the design of numerous anti-bleeding therapeutics that inhibit TFPI activity, including an anti-TFPI aptamer (BAX499) and several anti-TFPI monoclonal antibodies[45,48]. BAX499 was shown to increase clot formation in a non-human primate model of HA, as

observed by a shortening of time to clot in thromboelastography experiments[48]. Despite promising results in pre-clinical *in vivo* studies, BAX499 caused a paradoxical increase in bleeding in treated individuals, resulting in its withdrawal[49]. Concizumab, a humanized monoclonal antibody against TFPI, promoted increased thrombin generation in haemophilic plasma and decreased bleeding in PWH[45,50] but a recent phase III trial was paused by Novo Nordisk due to the occurrence of thrombotic events in several PWH treated with concizumab. A number of other additional anti-TFPI antibodies, namely PF-06741086 and BAY-1093884, are currently in early stages of clinical development[51,52].

The completion of the Human Genome Project in 2003 provided researchers with powerful research tools to identify genetic variants that contribute to disease. Genome-wide association studies (GWAS) involve screening genomes of large patient cohorts for genetic variations which are common across disease groups, in order to identify deleterious or protective gene variants. There are currently no GWAS reported for HA, largely due to the requirement for large patient cohort sizes of >1000 patients. In contrast, GWAS have successfully identified novel genes proposed to modulate factors that contribute to other bleeding disorders, such as low von Willebrand factor levels (VWF) levels in people with von Willebrand disease (VWD)[53]. This study suggests a GWAS on a large cohort of PWH to identify novel genes that determine bleeding phenotype in severe PWH would be of significant value. An alternative approach to achieving this goal is whole-exome sequencing (WES), which examines only genomic protein coding regions and can be performed on much smaller patient cohorts[54,55]. An exemplar study carried out on PWH with anti-FVIII inhibitors to identify genetic contributors to risk of inhibitor development successfully identified a number of immunoregulatory genes that were associated with inhibitor development in PWH. Similar strategies to delineate the genetic bases for bleeding phenotype variability in PWH have the potential to identify novel loci for further investigation.

Subtle, cumulative variation(s) within biochemical and cellular haemostatic pathways may also contribute to bleeding phenotype variability in PWH. Various studies have utilised assays to evaluate global haemostatic activity as a method to predict bleeding risk. The most well-established of these assays is calibrated automated thrombinography, or thrombin generation assays (TGAs). One kinetic parameter measured during standard TGAs, the endogenous thrombin potential (ETP), correlates well with FVIII/FIX levels[56-58]. Notably, however, plasma from PWH with the same *F8* or *F9* mutation often exhibit significantly different TGA results, further reinforcing the notion that plasma FVIII/FIX levels are not always reliable predictors of

clinical bleeding phenotype[56,59]. Furthermore, although conceived as a global measure of blood coagulation capacity, the TGA is also unable to accurately characterise other aspects of haemostatic pathways whose activity is largely dependent upon endothelial cell function for normal activity, such as the protein C and TFPI anticoagulant pathways[60].

Inter-individual differences in fibrinolytic activity as a potential source of bleeding variability

Recent studies have alluded to a potentially important role for variable fibrinolytic activity in determining bleeding phenotype in PWH[8]. The fibrinolytic pathway consists broadly of two main stages; (1) plasmin generation and (2) the degradation of fibrin by plasmin. Plasminogen activation to plasmin is mediated by tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA)[61]. Fibrin helps facilitate its own degradation by binding tPA and plasminogen on its surface, leading to plasmin generation in close proximity to the clot[62]. Fibrin degradation products (FDPs) are released following plasmin-mediated fibrin degradation. Cleavage of fibrin exposes carboxy-terminal lysine residues which tPA and plasminogen can bind to, to promote further plasmin generation and clot degradation[61,63]. Fibrinolysis is regulated by proteases inhibitors that include plasminogen activator inhibitor 1 (PAI-1), α_2-antiplasmin (α_2-AP) and thrombin-activated fibrinolysis inhibitor (TAFI)[61,64-66].

Whereas global assays to detect functional defects in blood coagulation caused by haemophilia are well-established, these assays are unable to characterise concurrent alterations in fibrinolytic activity. Previous studies have indicated that hypofibrinolytic PWH may exhibit diminished bleeding tendency compared to other PWH. One previous study reported elevated tPA concentrations in a group of PWH compared to another PWH cohort with milder bleeding tendency[67], suggesting one determinant of clinical bleeding phenotype may be influenced by a lower threshold for plasminogen activation, a key step in fibrinolysis. Currently, no studies have investigated the role played by uPA in modulating individual bleeding phenotypes. The balance between tPA and PAI-1 levels in PWH is not well characterized, but it is conceivable that the regulatory relationship between these two components may become destabilised during a bleeding event. The role of PAI-1 in PWH remains unclear, but it has been shown in VWD that low levels of PAI-1 are associated with an increase in severity of clinical bleeding phenotype[68] and it remains to be determined whether this is also the case in PWH. α_2-AP deficiency results in accelerated fibrinolysis and excessive bleeding[69]; however, whether α_2-AP levels vary between individual PWH has yet to be fully investigated. In contrast, a recent study in which mice deficient in both plasminogen

and FVIII (*Plg*$^{-/-}$/*F8*$^{-/-}$) were generated found that these mice retained their bleeding phenotype and deficiency in plasminogen did not counter-balance defective haemostasis caused by FVIII deficiency in these mice[70].

TAFI, an important inhibitor of the fibrinolysis pathway, is activated to TAFIa by thrombin. Thrombin bound to its receptor thrombomodulin (TM) accelerates TAFI activation 1250-fold compared to thrombin alone[71]. Reduced thrombin generation in PWH results in less TAFI being activated by the thrombin-TM complex, impairing down-regulation of fibrinolytic activity[67,72]. Plasma TAFIa levels have been shown to be correlated with FVIII activity and TAFIa levels correspond well to bleeding phenotype, with decreased TAFIa resulting in a more intense bleeding pattern in PWH[73]. Pre-clinical studies have demonstrated that decreased clot lysis can be achieved by administering soluble TM to HA mice, which enhanced TAFI activation and thus downregulated fibrinolytic activity[74,75]. Conversely, APC generated by soluble TM is considered profibrinolytic due to its attenuation of thrombin generation and in turn reduced TAFI activation[76]. Moreover, APC has the capacity to degrade PAI-1 which would result in enhanced plasminogen activation[77]. Evidently, further examination of the cumulative activity of fibrinolytic enzymes and inhibitors is required to enhance our understanding of the contribution of altered fibrinolysis in determining phenotypic heterogeneity in PWH.

Cellular basis of bleeding phenotype variability in PWH?

Differences in platelet function between PWH may play a role in phenotypic heterogeneity. In 1973, Walsh *et al* reported that PWSH with a mild bleeding phenotype had elevated platelet activity compared to other PWSH[78]. A number of studies have demonstrated the importance of platelet activity for robust thrombin generation[79,80], with the influence of platelets most prevalent at very low levels of FVIII or FIX in PWSH[81]. Additionally, studies have investigated specific roles for altered platelet activation and aggregation in modulating bleeding tendency, but there exists conflicting information as to whether inter-individual differences in platelet activity contribute to variable bleeding tendency in PWH[18,82,83].

The endothelium is a critical interface between blood and the surrounding tissues[84] and is central to regulation of the coagulation cascade, inflammation and tissue repair[85]. There is significant heterogeneity in endothelial cell populations within vascular beds in different tissues within the body[85]. When vascular injury occurs, endothelial cells (ECs) become activated to release pro-coagulant, pro-thrombotic mediators[84]. The EC surface acts as a platform on which thrombin and TM assemble to activate protein C to APC and inhibit clot formation.

Interestingly, gene mutations that diminish endothelial surface TM expression are associated with increased bleeding tendency and could accentuate bleeding if co-inherited in affected PWH. TM-associated coagulopathy (TM-AC) is a recently recognised bleeding disorder caused by a mutation (p.Cys537Stop) in the *THBD* gene which results in shedding of soluble TM (sTM) from the endothelium into the circulation[86-88]. Consequently, high plasma sTM levels in these patients results in significantly increased APC generation leading to greatly reduced thrombin generation and disruption to clot formation. Additionally, this elevated plasma sTM can also mediate increased activation of TAFI resulting in delayed fibrinolysis despite a decrease in thrombin generation[86]. Given the distinct effects this p.Cys537Stop mutation can have on both the coagulation and fibrinolysis pathways, co-inheritance of a *THBD* gene mutation in a PWH could profoundly impact on their bleeding phenotype, although to date, there have been no reported cases of co-inheritance of the p.Cys537Stop mutation in PWH. Given the breadth of contributions made by the endothelial cell surface to regulation of clot size, fibrinolysis and tissue repair, it is feasible that inter-individual differences in endothelial cell expression patterns could impact on bleeding phenotype in PWH, however, there remains little research on the impact of differences in endothelial cell behaviour in PWH, and a dearth of convenient assays in which to investigate their role in modulating bleeding phenotype in PWH.

The future: variable inter-individual responses to gene therapy in PWH

Gene therapy offers the possibility of long-term correction of haemophilia in PWH from a single, one-off treatment. Adeno-associated viral (AAV) vectors encoding either FIX or FVIII cDNA have been used to deliver functional *F8* or *F9* genes for hepatic transgene expression[89]. Initial clinical trials demonstrated that a single intravenous infusion of AAV vector resulted in a prolonged and sustained increase in FVIII or FIX expression, that was still observed 1-year post-administration[90-92]. This resulted in the majority of treated participants discontinuing their prophylactic factor replacement, with no adverse effect on their annual bleed rate. One multicentre 5 year follow up study on HA participants showed that even 5 years post infusion, significantly increased plasma FVIII levels remained and almost all of the PWH had ceased routine clotting factor replacement injections[93]. Notably, clear differences between treated PWH were observed. A number of participants in this study continued to express FVIII at low levels, despite receiving the same dose as other participants. Furthermore, differences in inter-individual response in relation to gene therapy side effects have emerged, perhaps the most challenging of which is that ~40% of the general population have pre-existing immunity against AAV viral capsids[94]. The nature and extent of the immune response to AAV depends

on a number of factors including the dosage used for the infusion and the AAV serotype. In order to circumvent this issue, a short treatment with immunosuppressive steroids can be administered to encourage immune tolerance to the AAV capsid and lower doses of gene therapy used to prevent hepatotoxicity[95,96]. This potential increase in hepatoxicity means that current liver directed gene therapy would not be suitable for any PWH diagnosed with hepatitis, precluding them from this treatment approach[97]. Gene therapy is rightly perceived as as transformative therapy for long-lasting resolution of HA and HB bleeding, but there remains a requirement for better understanding of the determinants of inter-individual responses to gene therapy in PWH. Additionally, given the evidence presented above that other mechanisms independent of FVIII/FIX may be responsible for determining bleeding phenotype, gene therapy aimed at normalising deficient factor levels may not represent a definitive solution for all PWH.

Conclusions

Categorisation of PWH based on their endogenous factor levels often correlates well with their bleeding tendency and their likelihood to develop bleeding-related joint disease. Nevertheless, there remains a significant number of PWH who exhibit bleeding phenotypes that poorly correlate with their factor levels. Despite this, there exists a limited understanding of the biological basis of what dictates inter-individual variability in bleeding tendency in PWH. Time of first bleeding event or timing of administration of first prophylactic factor replacement appears to correlate well with subsequent development of bleeding frequency. Moreover, co-inheritance of prothrombotic gene mutations in PWH appears to aid in re-balancing haemostasis and therefore reduces bleeding tendency in these individuals. In addition, the potential role for variation in haemostatic pathways intertwined with thrombin generation, such as fibrinolysis, to nudge haemostasis either towards or away from balance, is increasingly apparent. Outstanding questions, however, remain as to whether cell-specific differences in either the vasculature or in circulating haemostatic or immune cells might impact upon bleeding phenotype, and large-scale genomic characterisation of PWH is likely to yield deeper, unexpected insights as to factors that modulate the bleeding phenotype of an individual with haemophilia. Enhanced understanding of the factors that modulate bleeding phenotype in PWH would have tangible benefits for the treatment of PWH and may accelerate the generation of diagnostic assays to enable a more granular insight into the bleeding tendency of PWH than is currently available. Characterisation of these biological parameters is likely to represent a critical step in enabling tailored treatments for PWH that prevent the occurrence of repeated bleeding events.

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Michael Dockal discloses conflict of interest as Takeda full time employee and has ownership of Takeda stock options and shares. The other authors have no conflicts of interest to declare.

Authorship

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References

1. Rodvien R, Mielke CH. Role of Platelets in Hemostasis and Thrombosis. Western Journal of Medicine 1976;125(3):181-186
2. Clemetson KJ. Platelets and Primary Haemostasis. Thrombosis Research 2012;129(3):220-224
3. Estevez B, Du X. New Concepts and Mechanisms of Platelet Activation Signaling. Physiology 2017;32(2):162-177
4. Dahlbäck B. Blood coagulation. The Lancet 2000;355(9215):1627-1632
5. Smith SA, Travers RJ, Morrissey JH. How it all starts: initiation of the clotting cascade. Critical reviews in biochemistry and molecular biology 2015;50(4):326-336
6. Dahlbäck B, Villoutreix BO. Regulation of Blood Coagulation by the Protein C Anticoagulant Pathway. Novel Insights Into Structure-Function Relationships and Molecular Recognition 2005;25(7):1311-1320
7. Hoyer LW. Hemophilia A. New England Journal of Medicine 1994;330(1):38-47
8. Franchini M, Mannucci PM. Modifiers of clinical phenotype in severe congenital hemophilia. Thromb Res 2017;156:60-64
9. A. AJ, M. M, F. Q, E. G, M. TJ. The orthopaedic status of severe haemophiliacs in Spain. Haemophilia 2000;6(3):170-176
10. M. AL, H. HR, H. P. A longitudinal study of orthopaedic outcomes for severe factor-VIII-deficient haemophiliacs. Journal of Internal Medicine 1994;236(4):391-399
11. E. S, E. MM, A. T, et al. Severe hemophilia with mild bleeding phenotype: molecular characterization and global coagulation profile. Journal of Thrombosis and Haemostasis 2010;8(4):737-743
12. Carcao MD, van den Berg HM, Ljung R, Mancuso ME. Correlation between phenotype and genotype in a large unselected cohort of children with severe hemophilia A. Blood 2013;121(19):3946-3952
13. Oldenburg J, Schroder J, Schmitt C, Brackmann HH, Schwaab R. Small deletion/insertion mutations within poly-A runs of the factor VIII gene mitigate the severe haemophilia A phenotype. Thromb Haemost 1998;79(2):452-453
14. Franchini M, Lippi G. Factor V Leiden and hemophilia. Thromb Res 2010;125(2):119-123
15. Nowak-Gottl U, Escuriola C, Kurnik K, et al. Haemophilia and thrombophilia. What do we learn about combined inheritance of both genetic variations? Hamostaseologie 2003;23(1):36-40
16. Nogami K, Shima M. Phenotypic heterogeneity of hemostasis in severe hemophilia. Seminars in thrombosis and hemostasis 2015;41(8):826-831
17. van den Berg HM, De Groot PH, Fischer K. Phenotypic heterogeneity in severe hemophilia. Journal of thrombosis and haemostasis : JTH 2007;5 Suppl 1:151-156
18. Riedl J, Ay C, Pabinger I. Platelets and hemophilia: A review of the literature. Thromb Res 2017;155:131-139
19. Hang MX, Blanchette VS, Pullenayegum E, McLimont M, Feldman BM. Age at first joint bleed and bleeding severity in boys with severe hemophilia A: Canadian Hemophilia Primary Prophylaxis Study. Journal of thrombosis and haemostasis : JTH 2011;9(5):1067-1069
20. Onwuzurike N, Warrier I, Lusher JM. Types of bleeding seen during the first 30 months of life in children with severe haemophilia A and B. Haemophilia 1996;2(3):137-140

21. Dijk K, Fischer K, Bom JG, Grobbee DE, Berg HM. Variability in clinical phenotype of severe haemophilia: the role of the first joint bleed. Haemophilia 2005;11(5):438-443
22. SANTAGOSTINO E, MANCUSO ME, TRIPODI A, et al. Severe hemophilia with mild bleeding phenotype: molecular characterization and global coagulation profile. Journal of Thrombosis and Haemostasis 2010;8(4):737-743
23. Kreuz W, Escuriola Ettingshausen C, Funk M, et al. Prevention of joint damage in hemophilic children with early prophylaxis. Der Orthopade 1999;28(4):341-346
24. Fischer K, van der Bom JG, Mauser-Bunschoten EP, et al. The effects of postponing prophylactic treatment on long-term outcome in patients with severe hemophilia. Blood 2002;99(7):2337-2341
25. Astermark J, Petrini P, Tengborn L, et al. Primary prophylaxis in severe haemophilia should be started at an early age but can be individualized. British Journal of Haematology 1999;105(4):1109-1113
26. Mannucci PM, Franchini M. Is haemophilia B less severe than haemophilia A? Haemophilia 2013;19(4):499-502
27. Castaman G, Matino D. Hemophilia A and B: molecular and clinical similarities and differences. Haematologica 2019;104(9):1702-1709
28. Iorio A, Fischer K, Blanchette V, et al. Tailoring treatment of haemophilia B: accounting for the distribution and clearance of standard and extended half-life FIX concentrates. Thromb Haemost 2017;117(6):1023-1030
29. Cheung WF, van den Born J, Kuhn K, et al. Identification of the endothelial cell binding site for factor IX. Proc Natl Acad Sci U S A 1996;93(20):11068-11073
30. Stafford DW. Extravascular FIX and coagulation. Thromb J 2016;14(Suppl 1):35
31. Tagariello G, Iorio A, Santagostino E, et al. Comparison of the rates of joint arthroplasty in patients with severe factor VIII and IX deficiency: an index of different clinical severity of the 2 coagulation disorders. Blood 2009;114(4):779-784
32. Melchiorre D, Linari S, Manetti M, et al. Clinical, instrumental, serological and histological findings suggest that hemophilia B may be less severe than hemophilia A. Haematologica 2016;101(2):219-225
33. Goodeve AC, Preston FE, Peake IR. Factor VIII gene rearrangements in patients with severe haemophilia A. Lancet (London, England) 1994;343(8893):329-330
34. Oldenburg J. Mutation profiling in haemophilia A. Thromb Haemost 2001;85(4):577-579
35. Belvini D, Salviato R, Radossi P, et al. Molecular genotyping of the Italian cohort of patients with hemophilia B. Haematologica 2005;90(5):635-642
36. Yu T, Dai J, Liu H, et al. Spectrum of F9 mutations in Chinese haemophilia B patients: identification of 20 novel mutations. Pathology 2012;44(4):342-347
37. Franchini M, Mannucci PM. Haemophilia B is clinically less severe than haemophilia A: further evidence. Blood Transfusion 2018;16(2):121-122
38. Mancuso ME, Chantarangkul V, Cannavò A, et al. Is Hemophilia B Less Severe Than Hemophilia A? Results Of Global Coagulation Assays. Blood 2013;122(21):2352-2352
39. van 't Veer C, Golden NJ, Kalafatis M, et al. An in vitro analysis of the combination of hemophilia A and factor V(LEIDEN). Blood 1997;90(8):3067-3072
40. Lee DH, Walker IR, Teitel J, et al. Effect of the factor V Leiden mutation on the clinical expression of severe hemophilia A. Thromb Haemost 2000;83(3):387-391

41. Schlachterman A, Schuettrumpf J, Liu JH, et al. Factor V Leiden improves in vivo hemostasis in murine hemophilia models. Journal of thrombosis and haemostasis : JTH 2005;3(12):2730-2737
42. Negrier C, Berruyer M, Durin A, Philippe N, Dechavanne M. Increased thrombin generation in a child with a combined factor IX and protein C deficiency. Blood 1993;81(3):690-695
43. Tardy-Poncet B, Piot M, Chapelle C, Berger C, Tardy B. Difference in TFPI levels between haemophilia A and B patients. Haemophilia 2011;17(2):312-313
44. Maroney SA, Cooley BC, Ferrel JP, et al. Absence of hematopoietic tissue factor pathway inhibitor mitigates bleeding in mice with hemophilia. Proc Natl Acad Sci U S A 2012;109(10):3927-3931
45. Waters EK, Sigh J, Friedrich U, Hilden I, Sorensen BB. Concizumab, an anti-tissue factor pathway inhibitor antibody, induces increased thrombin generation in plasma from haemophilia patients and healthy subjects measured by the thrombin generation assay. Haemophilia 2017;23(5):769-776
46. Polderdijk SGI, Baglin TP, Huntington JA. Targeting activated protein C to treat hemophilia. Current Opinion in Hematology 2017;24(5):446-452
47. Polderdijk SG, Adams TE, Ivanciu L, et al. Design and characterization of an APC-specific serpin for the treatment of hemophilia. Blood 2017;129(1):105-113
48. Waters EK, Genga RM, Schwartz MC, et al. Aptamer ARC19499 mediates a procoagulant hemostatic effect by inhibiting tissue factor pathway inhibitor. Blood 2011;117(20):5514-5522
49. Dockal M, Hartmann R, Knappe S, et al. Effect of Increased Tissue Factor Pathway Inhibitor (TFPI) Levels On Factor Xa Inhibition and Global Hemostasis in the Presence of TFPI-Antagonistic Aptamer BAX 499. Blood 2012;120(21):2207-2207
50. Shapiro AD, Angchaisuksiri P, Astermark J, et al. Subcutaneous concizumab prophylaxis in hemophilia A and hemophilia A/B with inhibitors: phase 2 trial results. Blood 2019;134(22):1973-1982
51. Hilden I, Lauritzen B, Sorensen BB, et al. Hemostatic effect of a monoclonal antibody mAb 2021 blocking the interaction between FXa and TFPI in a rabbit hemophilia model. Blood 2012;119(24):5871-5878
52. Chowdary P, Lethagen S, Friedrich U, et al. Safety and pharmacokinetics of anti-TFPI antibody (concizumab) in healthy volunteers and patients with hemophilia: a randomized first human dose trial. Journal of thrombosis and haemostasis : JTH 2015;13(5):743-754
53. van Loon J, Dehghan A, Weihong T, et al. Genome-wide association studies identify genetic loci for low von Willebrand factor levels. European journal of human genetics : EJHG 2016;24(7):1035-1040
54. Choi M, Scholl UI, Ji W, et al. Genetic diagnosis by whole exome capture and massively parallel DNA sequencing. Proc Natl Acad Sci U S A 2009;106(45):19096-19101
55. van Dijk EL, Auger H, Jaszczyszyn Y, Thermes C. Ten years of next-generation sequencing technology. Trends in Genetics 2014;30(9):418-426
56. Santagostino E, Mancuso ME, Tripodi A, et al. Severe hemophilia with mild bleeding phenotype: molecular characterization and global coagulation profile. Journal of thrombosis and haemostasis : JTH 2010;8(4):737-743

57. Trossaert M, Regnault V, Sigaud M, et al. Mild hemophilia A with factor VIII assay discrepancy: using thrombin generation assay to assess the bleeding phenotype. Journal of thrombosis and haemostasis : JTH 2008;6(3):486-493
58. Dargaud Y, Béguin S, Lienhart A, et al. Evaluation of thrombin generating capacity in plasma from patients with haemophilia A and B. Thromb Haemost 2005;93(03):475-480
59. Chantarangkul V, Clerici M, Bressi C, Giesen P, Tripodi A. Thrombin generation assessed as endogenous thrombin potential in patients with hyper- or hypo-coagulability. Haematologica 2003;88(5):547-554
60. P. B-MC, A. K, R. J-CA, A. LM. Thrombin generation and phenotypic correlation in haemophilia A. Haemophilia 2005;11(4):326-334
61. Cesarman-Maus G, Hajjar KA. Molecular mechanisms of fibrinolysis. Br J Haematol 2005;129(3):307-321
62. Medved L, Nieuwenhuizen W. Molecular mechanisms of initiation of fibrinolysis by fibrin. Thromb Haemost 2003;89(3):409-419
63. Chapin JC, Hajjar KA. Fibrinolysis and the control of blood coagulation. Blood Rev 2015;29(1):17-24
64. Sprengers E, Kluft C. Plasminogen activator inhibitors. Blood 1987;69(2):381-387
65. Nesheim M, Bajzar L. The discovery of TAFI. Journal of thrombosis and haemostasis : JTH 2005;3(10):2139-2146
66. Foley JH, Kim PY, Mutch NJ, Gils A. Insights into thrombin activatable fibrinolysis inhibitor function and regulation. Journal of thrombosis and haemostasis : JTH 2013;11 Suppl 1:306-315
67. Grunewald M, Siegemund A, Grunewald A, et al. Paradoxical hyperfibrinolysis is associated with a more intensely haemorrhagic phenotype in severe congenital haemophilia. Haemophilia 2002;8(6):768-775
68. Abdul S, Boender J, Malfliet JJMC, et al. Plasma levels of plasminogen activator inhibitor-1 and bleeding phenotype in patients with von Willebrand disease. Haemophilia 2017;23(3):437-443
69. Carpenter SL, Mathew P. Alpha2-antiplasmin and its deficiency: fibrinolysis out of balance. Haemophilia 2008;14(6):1250-1254
70. Stagaard R, Ley CD, Almholt K, et al. Absence of functional compensation between coagulation factor VIII and plasminogen in double-knockout mice. Blood advances 2018;2(22):3126-3136
71. Bajzar L, Morser J, Nesheim M. TAFI, or plasma procarboxypeptidase B, couples the coagulation and fibrinolytic cascades through the thrombin-thrombomodulin complex. J Biol Chem 1996;271(28):16603-16608
72. Broze GJ, Jr., Higuchi DA. Coagulation-dependent inhibition of fibrinolysis: role of carboxypeptidase-U and the premature lysis of clots from hemophilic plasma. Blood 1996;88(10):3815-3823
73. Foley JH, Nesheim ME, Rivard GE, Brummel-Ziedins KE. Thrombin activatable fibrinolysis inhibitor activation and bleeding in haemophilia A. Haemophilia 2012;18(3):e316-322
74. Foley JH, Nesheim ME. Soluble thrombomodulin partially corrects the premature lysis defect in FVIII-deficient plasma by stimulating the activation of thrombin activatable fibrinolysis inhibitor. Journal of thrombosis and haemostasis : JTH 2009;7(3):453-459

75. Mosnier LO, Lisman T, van den Berg HM, et al. The defective down regulation of fibrinolysis in haemophilia A can be restored by increasing the TAFI plasma concentration. Thromb Haemost 2001;86(4):1035-1039
76. Mosnier LO, Bouma BN. Regulation of fibrinolysis by thrombin activatable fibrinolysis inhibitor, an unstable carboxypeptidase B that unites the pathways of coagulation and fibrinolysis. Arterioscler Thromb Vasc Biol 2006;26(11):2445-2453
77. Sakata Y, Loskutoff DJ, Gladson CL, Hekman CM, Griffin JH. Mechanism of protein C-dependent clot lysis: role of plasminogen activator inhibitor. Blood 1986;68(6):1218-1223
78. Walsh PN, Rainsford SG, Biggs R. Platelet coagulant activities and clinical severity in haemophilia. THROMBDIATHESHAEMORRH 1973;29(3):722-729
79. Herault JP, Peyrou V, Savi P, Bernat A, Herbert JM. Effect of SR121566A, a potent GP IIb-IIIa antagonist on platelet-mediated thrombin generation in vitro and in vivo. Thromb Haemost 1998;79(2):383-388
80. Wegert W, Graff J, Kaiser D, et al. Effects of antiplatelet agents on platelet-induced thrombin generation. International journal of clinical pharmacology and therapeutics 2002;40(4):135-141
81. Siegemund T, Petros S, Siegemund A, Scholz U, Engelmann L. Thrombin generation in severe haemophilia A and B: the endogenous thrombin potential in platelet-rich plasma. Thromb Haemost 2003;90(5):781-786
82. van Bladel ER, Roest M, de Groot PG, Schutgens REG. Up-regulation of platelet activation in hemophilia A. Haematologica 2011;96(6):888-895
83. Jensen MS, Larsen OH, Christiansen K, et al. Platelet activation and aggregation: the importance of thrombin activity--a laboratory model. Haemophilia 2013;19(3):403-408
84. Yau JW, Teoh H, Verma S. Endothelial cell control of thrombosis. BMC Cardiovascular Disorders 2015;15
85. van Hinsbergh VWM. Endothelium—role in regulation of coagulation and inflammation. Seminars in Immunopathology 2012;34(1):93-106
86. Burley K, Whyte CS, Westbury SK, et al. Altered fibrinolysis in autosomal dominant thrombomodulin-associated coagulopathy. Blood 2016;128(14):1879-1883
87. Dargaud Y, Scoazec JY, Wielders SJ, et al. Characterization of an autosomal dominant bleeding disorder caused by a thrombomodulin mutation. Blood 2015;125(9):1497-1501
88. Langdown J, Luddington RJ, Huntington JA, Baglin TP. A hereditary bleeding disorder resulting from a premature stop codon in thrombomodulin (p.Cys537Stop). Blood 2014;124(12):1951-1956
89. Perrin GQ, Herzog RW, Markusic DM. Update on clinical gene therapy for hemophilia. Blood 2019;133(5):407-414
90. Rangarajan S, Walsh L, Lester W, et al. AAV5–Factor VIII Gene Transfer in Severe Hemophilia A. New England Journal of Medicine 2017;377(26):2519-2530
91. George LA, Sullivan SK, Giermasz A, et al. Hemophilia B Gene Therapy with a High-Specific-Activity Factor IX Variant. The New England journal of medicine 2017;377(23):2215-2227
92. Miesbach W, Meijer K, Coppens M, et al. Gene therapy with adeno-associated virus vector 5-human factor IX in adults with hemophilia B. Blood 2018;131(9):1022-1031

93. Pasi KJ, Rangarajan S, Mitchell N, et al. Multiyear Follow-up of AAV5-hFVIII-SQ Gene Therapy for Hemophilia A. The New England journal of medicine 2020;382(1):29-40
94. Hurlbut GD, Ziegler RJ, Nietupski JB, et al. Preexisting immunity and low expression in primates highlight translational challenges for liver-directed AAV8-mediated gene therapy. Molecular therapy : the journal of the American Society of Gene Therapy 2010;18(11):1983-1994
95. Nathwani AC, Reiss UM, Tuddenham EG, et al. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. The New England journal of medicine 2014;371(21):1994-2004
96. Samelson-Jones BJ, Arruda VR. Protein-Engineered Coagulation Factors for Hemophilia Gene Therapy. Molecular therapy Methods & clinical development 2019;12:184-201
97. Doshi BS, Arruda VR. Gene therapy for hemophilia: what does the future hold? Therapeutic advances in hematology 2018;9(9):273-293

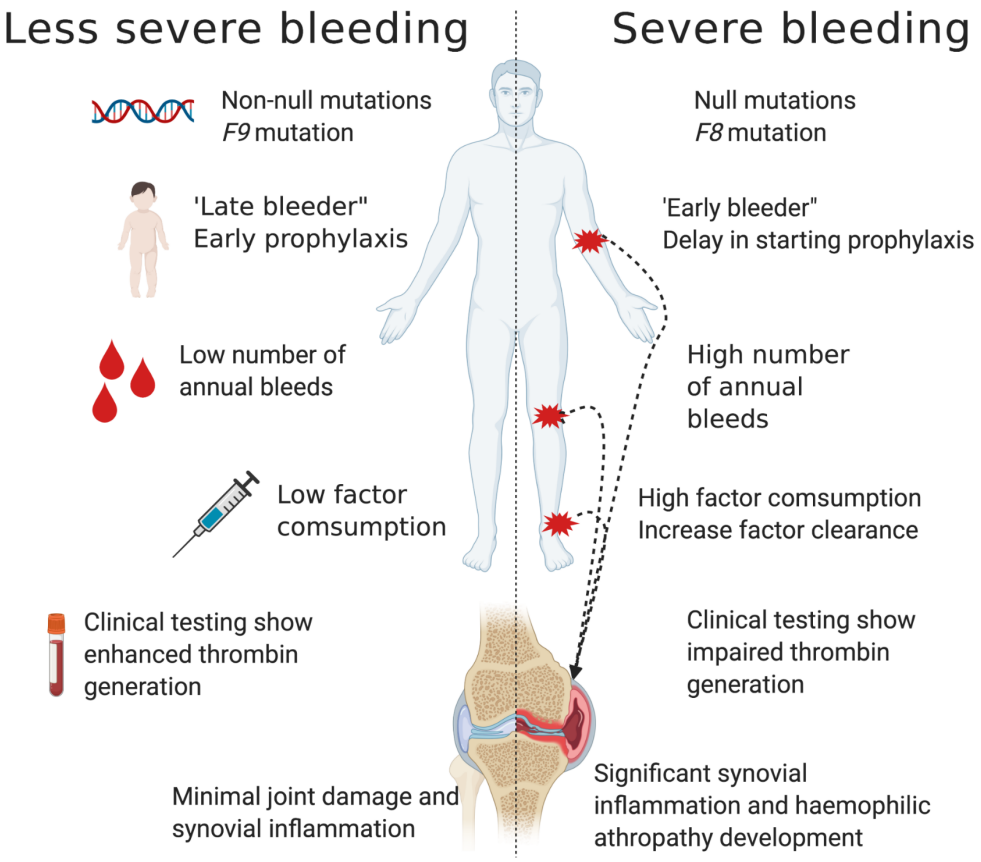

Figure 1: Determinants of bleeding heterogeneity in PWH. In addition to their endogenous factor levels, a number of factors have been shown to impact on the phenotypic heterogeneity in PWH. Those with less severe bleeding phenotypes will, in general, have a non-null causative mutation, low number of annual bleeds, have a lower requirement for factor replacement therapy, will experience their first joint bleed at an older age and will have minimal joint damage. PWH that start prophylactic replacement therapy at a young age have been shown to exhibit a less severe bleeding phenotype. Additionally, people with haemophilia B often have less intense bleeding phenotypes than those individuals with haemophilia A, although the biological mechanisms underlying this discrepancy remain incompletely understood.

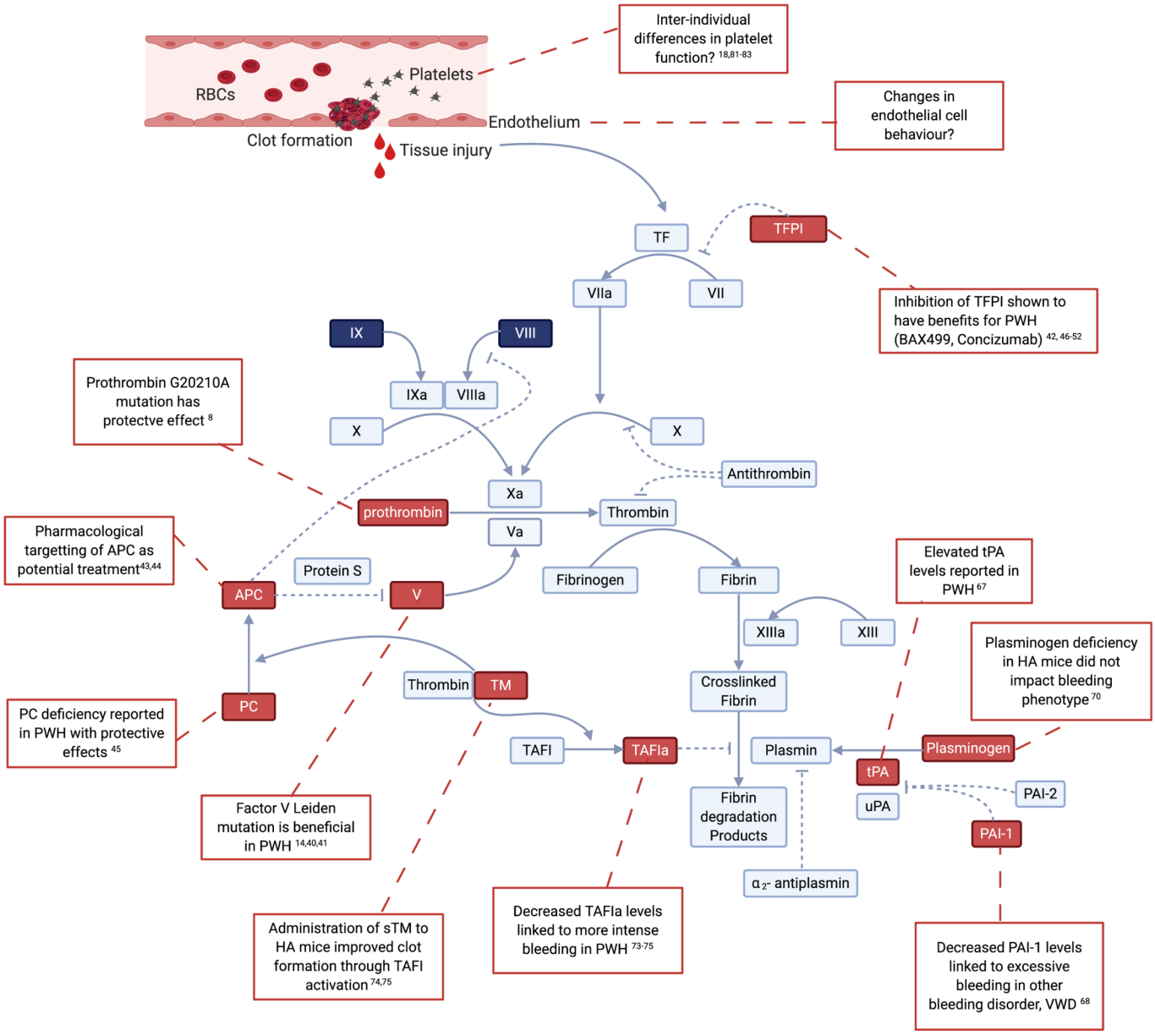

Figure 2: Variation of coagulation and fibrinolysis pathways that may contribute to bleeding heterogeneity between PWH. Co-inheritance of mutations in other coagulation proteins are associated with reduced bleeding tendency in PWH. Prothrombin G20210A mutation, Factor V Leiden and Protein C (PC) deficiency all allow for more robust clot formation, either through promotion of thrombin generation or by inhibition of anticoagulant pathways. As a consequence, pharmacological targeting of anticoagulant activated PC (APC) and Tissue Factor Pathway Inhibitor (TFPI) has been postulated to reduce bleeding in PWH. Soluble Thrombomodulin (sTM) administration promotes clot formation through activation of TAFI and downregulation of the fibrinolytic pathway while low levels of plasma TAFI correlates with severe bleeding in PWH. Elevated tissue Plasminogen Activator (tPA) has been observed in PWH however current knowledge on the role of PAI-1 in mitigating bleeding tendency in PWH remains unclear. It is also possible that inter-individual differences in unknown factors that control cellular activation and functions in platelets and endothelial cells could also contribute to the bleeding heterogeneity observed in PWH, although this currently remains largely unexplored.

Table 1: Biological factors which may influences severity of bleeding phenotype in PWH

Factor influencing bleeding phenotype	References
Age at first joint bleed	21,22
Age to begin prophylactic factor replacement	23,25
Differences in severity between HA and HB	26,27
Null versus non-null mutation	11,33,35
Co-inheritance of a prothrombotic mutation	8, 40 - 44
Inter-individual differences in fibrinolysis	68, 74, 77
Inter-individual differences in cellular function	81, 86
Heterogeneity in response to gene therapy	94, 96