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Genetics of equine bleeding disorders

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Summary

Genetic bleeding disorders can have a profound impact on a horse's health and athletic career. As such, it is important to understand the mechanisms of these diseases and how they are diagnosed. These diseases include haemophilia A, von Willebrand disease, prekallikrein deficiency, Glanzmann's Thrombasthenia, and Atypical Equine Thrombasthenia. Exercise-induced pulmonary haemorrhage also has a proposed genetic component. Genetic mutations have been identified for haemophilia A and Glanzmann's Thrombasthenia in the horse. Mutations are known for von Willebrand disease and prekallikrein deficiency in other species. In the absence of genetic tests, bleeding disorders are typically diagnosed by measuring platelet function, von Willebrand factor, and other coagulation protein levels and activities. For autosomal recessive diseases, genetic testing can prevent the breeding of two carriers.

Keywords

horse; haemophilia A; von Willebrand disease; prekallikrein deficiency; Glanzmann's Thrombasthenia; Atypical Equine Thrombasthenia

Background

Clotting is an essential biologic process. Several genetic bleeding disorders have been identified in horses, affecting the coagulation cascade and platelet function. These disorders are typically heritable and therefore passed down from parent to offspring. Genetic mechanisms have been identified for some equine bleeding disorders while others remain

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Authorship

A. Dahlgren drafted the manuscript and revised it. F. Tablin and C. Finno substantively revised the manuscript.

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No competing interests have been declared.

Ethical animal research

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Owner informed consent

Not applicable.

Data accessibility statement

Not applicable as no new data were generated.

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unknown. This review is focused on the genetics of equine diseases affecting the coagulation cascade and platelet function.

Haemostasis Testing

Haemostasis tests are the cornerstone of diagnosing bleeding disorders across species. To appreciate how bleeding disorders in horses are diagnosed, it is necessary to understand how several of these tests work. Platelet count is important as it can indicate an ongoing disease or infection if the count is higher or lower than the normal range (94-232 x 10^3/uL at Cornell Clinical Pathology Laboratory). In addition to assessing overall platelet number on a complete blood count, a basic large animal coagulation panel, which includes activated partial thromboplastin time (aPTT), prothrombin time (PT), and fibrinogen should be evaluated. The first two quantify the time for blood to clot following the addition of standard amounts of agonists to plasma. Blood is drawn into a tube that contains citrate which acts as an anticoagulant by binding all the extracellular calcium. Plasma is separated by centrifugation and removed. An excess of calcium is added to the plasma to allow it to clot. To determine aPTT, an activating substance for Factor XII (e.g. silica, celite, kaolin, ellagic acid) is added to simulate the intrinsic (also known as the contact) pathway [1]. To determine PT, tissue factor is added to activate the extrinsic pathway [1]. To measure fibrinogen activity, thrombin is added to plasma and the time it takes for a clot to form is optically measured and compared to a reference interval [1]. The amount of fibrinogen is also compared to a species-specific standard curve to quantify how much fibrinogen is present in a volume of blood [1]. It is important to also keep in mind that increased fibrinogen can also be indicative of inflammation, not just a bleeding disorder [2]. Some laboratories also include the test for fibrin degradation products (FDPs) or D-dimers that are the result of the breakdown of a clot. FDPs and D-dimers differ in that D-dimers include the smallest crosslinked dimers of fibrin degradation products, while FDP measures all degradation products. This is an important distinction because the crosslinking only occurs when a clot is formed while fibrinogen breakdown can happen in the absence of clot formation. As such, D-dimer levels are more informative of clotting. The tests for FDPs and D-dimers are similar, consisting of using latex particles covered with antibodies against FDPs or D-dimers [1]. If FDPs or D-dimers are present, they will bind to the antibodies, causing the latex particles to bind together [1]. The extent to which the latex particles aggregate is quantified [1]. FDP and D-dimers levels generally are not informative for diagnosing genetic bleeding disorders.

Additional haemostasis tests important for diagnosing bleeding disorders include protein concentration and activity assays. The most well-known are probably factor assays where coagulation factor levels are measured. The primary method of diagnosing haemophilia A in horses is measuring factor VIII (FVIII) coagulant activity (FVIII:C). In this assay, horse plasma is mixed in various standard quantities with plasma that is deficient in FVIII. The amount of time it takes for each mixture to clot is measured, plotted, and compared to a standard curve to determine the levels of FVIII present [1]. If below the reference interval (50–200% at Cornell Comparative Coagulation Laboratory), the horse is considered deficient. Another important protein for diagnosing bleeding disorders is von Willebrand Factor (vWF). Two tests are typically used to measure vWF levels and function. The total

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plasma vWF antigen (vWF:Ag) quantifies vWF using an enzyme-linked immunosorbent assay (ELISA). Anti-vWF antibodies are used to specifically bind to vWF. These antibodies are also conjugated to an enzyme that reacts with a subsequently added substrate to produce colour which is quantified. vWF levels can then be measured against a standard curve. A second test measuring vWF activity uses ristocetin cofactor (vWF:RCo). Ristocetin is an antibiotic that induces binding of vWF to platelet glycoprotein Ib-IX-V, resulting in platelet-platelet aggregation which is then measured with an aggregometer. The rate of aggregation can be used to determine the levels of vWF. High molecular weight vWF multimers are visualised using agarose gel electrophoresis which is important when diagnosing the subtype of vWD. The severity of signs and total level of vWF present are used to split vWD into three different types (types 1, 2, and 3). Additionally, type 2 segregates into four subtypes (A, B, M, N), depending on quantity of high molecular weight multimers and abnormal binding affinity.

Thromboelastography is a method to specifically investigate platelet function in whole blood. This method evaluates at the changes in the viscoelastic properties of whole blood during aggregation and fibrinolysis. Whole blood is placed in a reaction cup that oscillates along with an agonist (ex. kaolin) and calcium [3]. As the clot forms, tension is applied to a wire connected to a pin in the cup [3]. This tension is translated into an output trace. From this trace, multiple variables can be calculated, including the time it takes for clot to start forming, fibrinogen concentration, and strength of clot [3]. Lastly, a controversial method for testing platelet function *in vivo* is measuring template bleeding time (TBT). Briefly, this is done via a standard size shallow incision on the horses' forelimb and paper is used to absorb the blood [4]. The time from incision to when the bleeding stops is measured [4]. There is no standardised reference range for horses, and it has been reported that TBT has wide variability between healthy horses [5]. However, a horse that consistently has a highly prolonged TBT likely has a bleeding disorder.

The results from a basic coagulation panel as well as subsequent tests can provide directions for the next steps in a diagnosis and treatment plan.

Inherited equine disorders affecting the coagulation cascade

The coagulation cascade describes the series of physiological events, including enzymatic activation of protein and recruitment to sites of injury, within the vasculature leading to haemostasis. Inherited equine disorders affecting this cascade include haemophilia A and prekallikrein deficiency.

Haemophilia A:

Haemophilia A, caused by mutations in the *F8* gene that lead to FVIII deficiency, leads to recurrent bleeding. Haematomas are also often reported [6-8]. Under physiological conditions, in response to tissue injury, thrombin becomes activated through the clotting cascade which in turn cleaves FVIII into its active form (FVIIIa). Then, FVIIIa acts as a co-factor to factor IXa (FIXa), activating factor X (FX) leading to the cleavage of prothrombin into more thrombin which has positive feedback on several coagulation factors. In horses

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affected with haemophilia A, a severe deficiency in FVIII results in a decreased ability to maintain this cascade, so a clot does not properly form.

In horses with haemophilia A, aPTT is typically prolonged, PT is normal, and fibrinogen levels are decreased. However, as noted above, inflammation can increase fibrinogen activity, thus this should be taken into account during diagnosis [2]. The primary method of diagnosis is measuring FVIII:C. If the horse is deficient for FVIII, haemophilia A is the likely cause of abnormal bleeding. Horses that are carriers for haemophilia A typically have lower levels of FVIII, sometimes even below the reference range [7]. However, they do not have bleeding problems.

The mode of inheritance of haemophilia A is X-linked recessive since *F8* is on the X-chromosome. Affected females have two non-functional copies and males have one non-functional copy of the F8 gene. As such, haemophilia A is more common in males, with all of the published records in male horses [6,9-14]. Haemophilia A has been reported in Thoroughbreds [7,10,11,15,16], Standardbreds [9,14], Quarter Horses [12,13], an Arabian [8], and a Tennessee Walker [6]. Other types of haemophilia (B and C) where other coagulation factors are deficient have been identified in humans [17,18], dogs [19-23], and cats [24]; however, haemophilia A is the only type that has been conclusively identified in horses. The haemophilic Arabian was reported to have deficiencies of other factors, though not to the extent that is usually seen in haemophilic individuals. While haemophilia A is the most common genetic bleeding disorder in horses, the genetic cause has only recently begun to be investigated. A Tennessee Walker affected with haemophilia A was found to have a four base pair (bp) deletion (EquCab3.0 chrX:127,502,317–127,502,314delAACA) and two linked single nucleotide polymorphisms (EquCab3.0 chrX:127,502,303G>C, chrX:127,502,320G>A) in intron 1 of *F8* [6]. This horse did not have detectable FVIII protein, though the antibody used to investigate it was not horse-specific and likely could not detect very low protein levels. However, it was also determined that exons 1-2 could not be amplified from *FVIII* mRNA, and it was hypothesised that the intron 1 variants affected splicing [6]. In people, haemophilia A is very heterogeneous, with almost 3,000 causative variants identified [25]. Thus, there are likely other undiscovered genetic causes for haemophilia A in the horse.

Prekallikrein deficiency:

Prekallikrein deficiency is a rare blood disorder in the horse. In healthy animals, factor XII (FXII) binds to the damaged endothelial surface and auto-activates to FXIIa. FXIIa then activates prekallikrein into plasma kallikrein, an additional activator of FXII, resulting in a positive feedback loop. FXIIa also plays a role in activating other coagulation factors leading to clot formation. Without functional prekallikrein, FXII is not activated as efficiently and can lead to abnormal bleeding. However, the phenotype can be quite subtle, as this is not the only pathway of coagulation factor activation.

Prekallikrein deficiency causes prolonged aPTT with normal PT and fibrinogen levels [26,27]. Prekallikrein levels are measured similarly to FVIII:C where patient plasma is mixed with prekallikrein-deficient plasma and the time to clot is measured. The times are compared to a standard curve to quantify the level of prekallikrein present.

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Prekallikrein deficiency has only been identified in two horse families, a Belgian family and a miniature horse family [26,27]. The initial Belgian horses bled excessively following castration and had normal levels of factors VIII, IX, XI, XII as well as a platelet count within the reference range [26]. However, prekallikrein deficiency does not always have obvious signs as in the case of the miniature horse [27]. The miniature horse was initially examined due to a metatarsophalangeal joint varus deformity. No other physical abnormalities were identified. Its blood failed to clot after 30 minutes, prompting further investigation and the identification of prekallikrein deficiency [27]. There was no history of abnormal bleeding [27]. A full sister to the miniature horse also had low levels of prekallikrein and no abnormal bleeding was reported [27]. Similarly, the Belgian that bled excessively had two full siblings with low levels of prekallikrein but no obvious coagulopathy [26]. This demonstrates that, similar to what is found in people, prekallikrein deficiency does not typically lead to a severe bleeding phenotype compared to the other haemostatic disorders. It may be important to note that neither of the reports of equine prekallikrein deficiency include information about platelet function (ex. thromboelastography) or vWF testing, possibly due to the resources available at the time of publication. Thus, there could be more going on than prekallikrein deficiency resulting in the abnormal bleeding in these families. While a genetic cause for prekallikrein deficiency has not been elucidated in the horse, causative variants have been identified in the *kallikrein B1* gene which encodes prekallikrein in people and dogs [28-32]. The mode of inheritance for prekallikrein deficiency has only been investigated in people, where it was shown to be inherited as an autosomal recessive trait [33]. Prekallikrein deficiency is also difficult to identify, likely due to normal clotting *in vivo*.

Inherited equine disorders affecting platelet function

Platelets are essential to the clotting process as these anucleate cells activate, adhere to the subendothelium, bind fibrinogen, and aggregate to form a clot. Without normal platelet function, blood cannot clot properly. Platelet function disorders identified in the horse include von Willebrand Disease (vWD), Glanzmann's Thrombasthenia (GT), and Atypical Equine Thrombasthenia (AET).

Von Willebrand disease (vWD):

vWD can result in epistaxis and abnormal bleeding after mild trauma or surgery. vWD is typically caused by mutations in the *von Willebrand Factor* gene, though acquired vWD has been reported rarely in humans and dogs [34-36]. The encoded protein (vWF) is a multimeric plasma glycoprotein that has several different roles. vWF binds to inactive FVIII to prevent degradation by activated protein C. vWF also binds to collagen that is exposed in damaged vascular sub-endothelium. Once vWF is bound to collagen, it can interact with platelets through the glycoprotein complex Ib-IX-V on the platelet membrane, which tethers the platelet to the site of injury. However, this interaction is not sufficient to make platelets firmly adhere to the site of injury. vWF binding to integrin $\alpha_{IIb}\beta_3$ provides a more stable platelet adhesion. Adenine diphosphate (ADP) and its receptors also play a necessary role in vWF-mediated adhesion. Adenine diphosphate is released from platelet dense granules as well as damaged red blood cells and can activate platelets by binding to receptors $P2Y_1$ and

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$P2Y_{12}$. Both receptors have been shown to be important in platelet aggregation after platelet adhesion to vWF bound to collagen [37]. Adenine diphosphate is also necessary for complete integrin $\alpha_{IIb}\beta_3$ activation and subsequent platelet adhesion [38]. Additionally, upregulated integrin $\alpha_{IIb}\beta_3$ binds to fibrinogen and engages in bidirectional signaling. While this signaling mediates platelet spreading, it also is regulated by glycoprotein VI (GPVI) and $\alpha_2\beta_1$. GPVI binds directly to collagen and is a major agonist for initial platelet activation. Integrin $\alpha_2\beta_1$ also is a major collagen receptor but requires inside-out activation. Another primary method of outside-in platelet activation is cleavage of a protease activated receptor on the platelet surface by thrombin which is quickly produced and activated by factor Xa, usually in a complex with factor V. Without sufficient levels or activity of vWF, platelets may not be able to protect FVIII from degradation, adhere to subendothelial collagen, or bind to platelet membrane proteins to tether platelets at the site of injury, leading to prolonged bleeding.

Horses affected with vWD may have mildly prolonged aPTT, but PT and fibrinogen are usually within normal ranges [39,40]. vWF:Ag and/or vWF:RCo will also be decreased depending on the type of vWD. When run on an agarose gel, the vWF may not be the correct size. vWD has been split into three types of which type 2 segregates into four subtypes to account for the variability of the disease. Type 1 describes a deficiency of vWF. In type 2 vWD, there is sufficient vWF, but it does not work properly. Subtype 2A indicates that the vWF multimers are not the correct size whereas subtype 2B indicates that vWF also is overly active. Subtype 2M describes vWF not being able to attach to the platelets. In subtype 2N, vWF does not bind properly to FVIII. Lastly, type 3 has very minimal levels of vWF.

vWD has been reported in two Quarter Horses [40,41] and a Thoroughbred mare and her colt [39]. The Quarter Horse filly [41] and the Thoroughbreds [39] were reported to have vWD type 2A characterised by decreased vWF activity and deficiency of high molecular weight vWF multimers. The Quarter Horse colt was hypothesised to have vWD type 1 due to signs of sufficient amounts of high molecular weight multimers [40]. However, formal subtyping was not performed [40]. No genetic mechanisms have been elucidated to date in the horse. There have been about 750 mutations identified in the human *vWF* gene associated with vWD [42] and at least four mutations associated with vWD in various dog breeds [43-46]. vWD has been observed to act in both a dominant and recessive mode of inheritance in humans depending on the specific mutation [42] and a recessive mode of inheritance in dogs [43-46]. Based on the few reports of vWD in horses, it does not appear to occur as frequently as it does in humans and dogs. To date, there are no studies on the mode of inheritance of vWD in the horse.

Glanzmann's Thrombasthenia (GT):

Hallmarks of GT include epistaxis and prolonged bleeding. GT is caused by mutations leading to a loss of function or deficiency of integrin αIIbβ3. This integrin is composed of two subunits encoded by two different genes (*ITGA2B* and *ITGB3*). A deleterious mutation in either gene leads to GT. Integrin αIIbβ3 is located on platelet membranes as well as on the platelet alpha granule membrane and binds fibrinogen, allowing activated platelets to

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aggregate and form a clot at the site of injury. With GT, there is either not enough integrin αIIbβ3 or αIIbβ3 does not properly bind to fibrinogen, inhibiting clot formation. In horses, there have only been reports of GT where there is an absence or very low expression of αIIbβ3.

The coagulation panel results in horses with GT remain within normal ranges. However, there is abnormal clot retraction and decreased platelet aggregation. Clot retraction is measured by drawing blood into a glass tube without anticoagulant and measuring the weight of the clot as well as the volume of remaining plasma upon complete contraction, compared with blood from control horses. It can also be assessed visually. Platelet aggregation, measured by an aggregometer, is determined by adding various agonists (e.g. adenine diphosphate [ADP], and collagen) to platelet-rich plasma. Thromboelastography has also been used as a diagnostic tool for GT. In one affected horse, clot formation time was increased and the clot was not as strong as a control horse [47]. While these tests can all suggest GT, they do not provide a conclusive diagnosis. Flow cytometry or western blot should be used to quantify αIIbβ3 protein levels with decreased protein expression indicating GT [47]. Ideally, a causative mutation identified in either *ITGA2B* and *ITGB3* would also confirm a GT diagnosis.

Two different mutations have been identified in the horse to cause GT. A missense mutation (Arg41Pro) in exon 2 of *ITGA2B* was identified in a Thoroughbred and an Oldenburg [48,49]. A 10 bp deletion in *ITGA2B* (EquCab3.0 chr11:19,247,983–19,247,992delCAGGTGAGGA) spanning the junction of exon 11 and intron 11 was identified as causative in a Peruvian Paso [50]. A GT-affected Quarter Horse was found to be a compound heterozygote for both of these variants [51]. Family studies have not been performed to look at the mode of inheritance of GT in horses, though GT has been observed to have a recessive mode of inheritance in humans [52]. Based on the parental and sibling genotypes included in the literature, it appears that horses have the same mode of inheritance [48,51].

Atypical Equine Thrombasthenia (AET):

AET is caused by abnormal platelet signaling leading to epistaxis and abnormal clotting after injury [53,54]. While pedigree analysis indicates that AET is heritable, the genetic cause has not yet been elucidated. The biochemical differences, however, have been thoroughly investigated. A number of proteins in the thrombin signaling pathway are decreased in quantity or activity in affected horses [53]. The biochemical hallmark of AET is that thrombin stimulated platelets from affected horses do not activate normally and bind fibrinogen less efficiently [55]. However, they do respond normally to other agonists (ADP) [55]. This is in contrast to GT where platelet aggregation is absent in response to all agonists.

An AET-affected horse was reported to have normal aPTT and slightly decreased PT [54]. Some factor activity was below the reference range, but this is more likely a result of long-term bleeding than indicative of factor deficiency. Additionally, the TBT was increased [54]. Currently, there is no readily available test that can be used to diagnose AET. It must be diagnosed via a fibrinogen-binding assay that uses thrombin as the stimulant after platelets

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have been confirmed to respond normally to ADP [55]. However, this test has high inter- and intra-individual variability, indicating the need for a more precise diagnostic tool (unpublished, Tablin lab).

The first horse diagnosed with AET was a mare and identified when she could not clot after pin firing. Subsequently, one of her offspring was diagnosed with the disease and her other offspring had inconclusive results with the fibrinogen-binding assay [55]. At this time, AET has only been identified in Thoroughbreds. Within a single breeding and training farm, AET had a prevalence of every one in 150 Thoroughbreds [56].

Other bleeding disorders

Exercise-induced pulmonary haemorrhage (EIPH):

EIPH describes any haemorrhage in the lungs, typically resulting from intense physical exercise. It is generally thought to be caused by a cyclic pattern of increased pulmonary vascular pressures leading to thickening of pulmonary vein walls and vice versa. Together, this leads to pulmonary capillary failure and blood leaking into the interstitial and alveolar spaces in the lung. However, while it is not always consistent [57], some have shown in Thoroughbreds and Standardbreds that platelets in EIPH-affected horses may not be as responsive to ADP stimulation compared to control horses [58,59]. Thus, there may be an unknown mechanism involving decreased platelet function in affected horses. Horses that already have decreased coagulation signaling or platelet dysfunction from an inherited disorder are likely at a higher risk for haemorrhagic disorders, including EIPH. EIPH has a high frequency in Thoroughbreds (44 [60] −75% [61]), Standardbreds (87% [62]), and Quarter horses (62.3% [63]). The heritability of EIPH has been investigated in Thoroughbreds and found to be between 0.23 and 0.5, depending on the population and model used [64,65]. In these retrospective studies, EIPH was defined as presence of blood in nostrils. EIPH is also diagnosed via endoscopy and bronchoalveolar lavage as haemorrhaging in the lungs is not always severe enough to cause epistaxis. A small (n=6) study in Standardbreds indicated that PT and aPTT are all within the reference range [66]. One complication of EIPH diagnoses is that other potential causes for haemorrhaging should be excluded. Haemophilia A, vWD, GT, and AET have all been reported to cause epistaxis [6,8,39,48,50,54] and potentially contribute to the previously determined heritability of EIPH. While most of these bleeding disorders would likely prevent a horse from becoming a successful racehorse, AET has not been reported to have as severe of a phenotype. Thus, if truly at a high frequency [56], AET could potentially influence the calculated EIPH heritability. However, additional research is necessary to further elucidate the role genetics and platelet function play in EIPH.

Conclusion

Abnormal bleeding can arise from coagulation factor deficiency, decreased platelet function, strenuous exercise, and primary medical conditions. Genetic variants can lead to factor deficiencies, abnormal platelet function, and potentially increase risk of bleeding during exercise. To date, causative genetic mutations have only been identified for haemophilia A and GT in the horse. However, the genetic mechanisms for vWD and prekallikrein

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deficiency are known in humans and dogs. Similar future discoveries will therefore likely be made in horses. The genetic mechanism for AET remains unknown. These inherited bleeding diseases may contribute to the risk for EIPH, and EIPH has been shown to potentially have a heritable component. However, additional studies are required to understand the interaction between genetics and EIPH.

Coagulation, platelet function, and molecular tests are currently used to diagnose bleeding disorders either through direct tests of the coagulation factors or by process of elimination. Identified carriers of autosomal recessive traits should not be bred to other carriers. Ideally, the mutation causing the bleeding disease in the affected offspring should be identified and a genetic test developed to assist in making breeding decisions.

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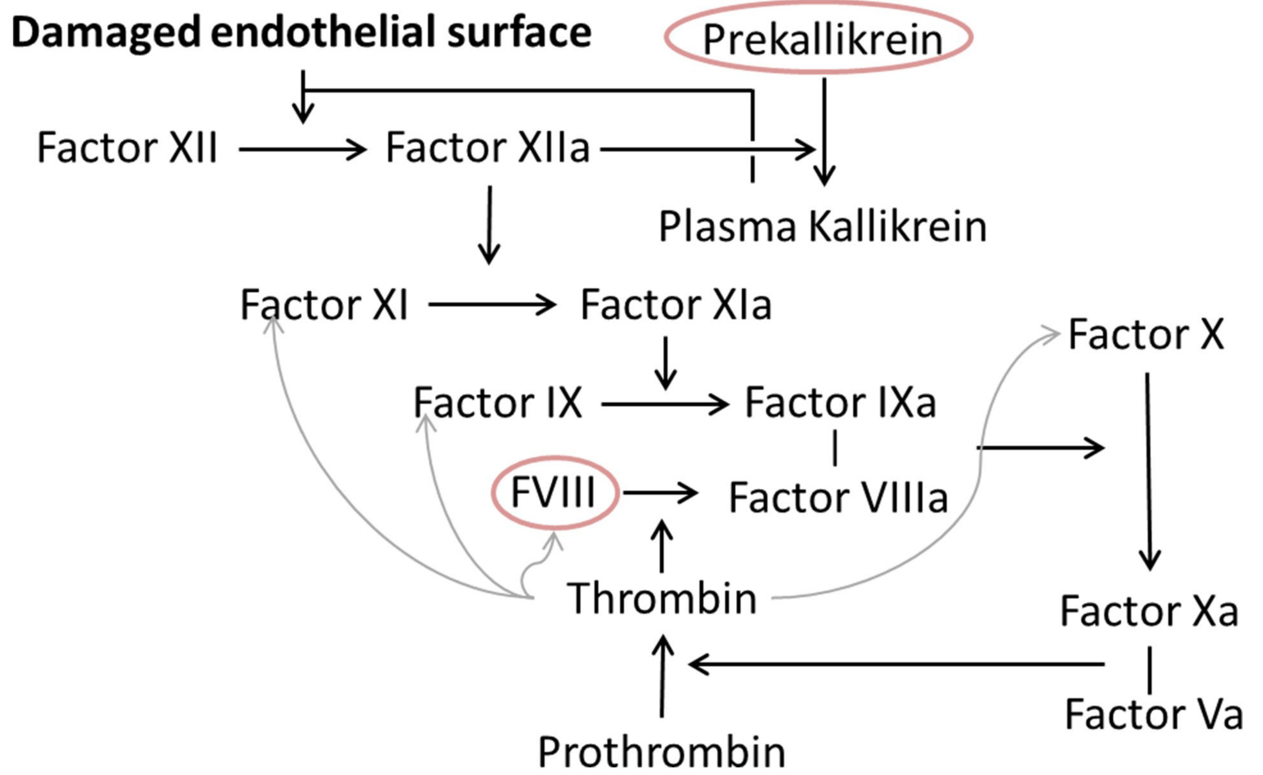

Fig 1:
Schematic of coagulation factor signaling pathway affected by prekallikrein deficiency and haemophilia A. Red ovals indicate the step in the pathway where each disease affects coagulation signaling. Grey arrows indicate thrombin positive feedback.

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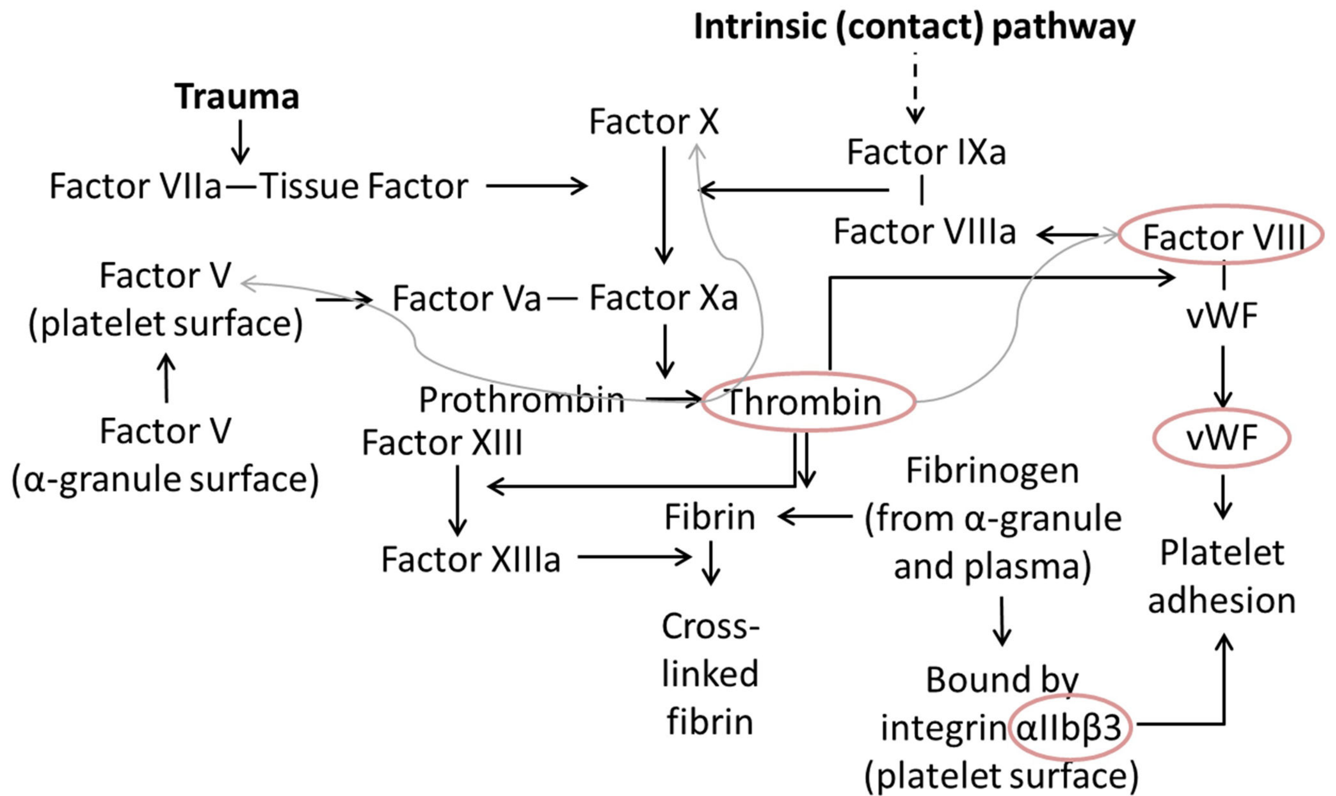

Fig 2:

Schematic of coagulation factor and platelet signaling pathways affected by vWD, GT, and AET. Red ovals indicate the step in the pathway where each disease affects coagulation signaling. Grey arrows indicate thrombin positive feedback.

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Table 1:

Summary of bleeding disorders with a genetic component.

Disease	Clinical signs	Diagnostic findings
Haemophilia A	Recurrent bleeding, haematomas	Prolonged aPTT, highly deficient FVIII:C, decreased fibrinogen, normal PT, normal FDP/d-dimers
Prekallikrein deficiency	Abnormal bleeding, often asymptomatic	Prolonged aPTT, deficient prekallikrein levels, normal PT, normal FDP/d-dimers
von Willebrand disease	Epistaxis, abnormal bleeding after trauma or surgery	Decreased vWF:Ag, decreased vWF:RC, smaller multimers, mildly prolonged aPTT, normal PT, normal FDP/d-dimers
Glanzmann's Thrombasthenia	Epistaxis, prolonged bleeding	Abnormal clot retraction, decreased platelet aggregation to all agonists, increased clot formation time, decreased clot strength, decreased protein expression of αIIbβ3, normal PT, normal aPTT, normal FDP/d-dimers
Atypical Equine Thrombasthenia	Epistaxis, abnormal bleeding after injury	Increased TBT, abnormal platelet response to thrombin stimulation, slightly decreased PT, normal aPTT, normal FDP/d-dimers
Exercise-induced pulmonary haemorrhage	Epistaxis, poor performance	Visual inspection, endoscopy, bronchoalveolar lavage, normal PT, normal aPTT, normal FDP/d-dimers