# Mechanism, Functions, and Diagnostic Relevance of FXII Activation by Foreign Surfaces

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#### Abstract

**Keywords** 

# Factor XII (FXII) is a serine protease zymogen produced by hepatocytes and secreted into plasma. The highly glycosylated coagulation protein consists of six domains and a proline-rich region that regulate activation and function. Activation of FXII results from a conformational change induced by binding ("contact") with negatively charged surfaces. The activated serine protease FXIIa drives both the proinflammatory kallikrein-kinin pathway and the procoagulant intrinsic coagulation cascade, respectively. Deficiency in FXII is associated with a prolonged activated partial thromboplastin time (aPTT) but not with an increased bleeding tendency. However, genetic or pharmacological deficiency impairs both arterial and venous thrombosis in experimental models. This review summarizes current knowledge of FXII structure, mechanisms of FXII contact activation, and the importance of FXII for diagnostic coagulation testing and thrombosis.

contact systemblood coagulation

polyphosphate

inflammation

factor XII

# Zusammenfassung

# Faktor XII (FXII) ist das Zymogen der Serinprotease FXIIa, wird von Hepatozyten produziert und ins Plasma sekretiert. Das Plasmaprotein besteht aus sechs Domänen sowie einer Prolin-reichen Region und ist stark glykosyliert. Die Aktivierung von FXII erfolgt durch eine Konformationsänderung, die durch die Bindung (den "Kontakt") mit negativ geladenen Oberflächen hervorgerufen wird. In der Folge aktiviert FXIIa sowohl das proinflammatorische Kallikrein-Kinin-System, welches Bradykinin produziert, als auch die intrinsische Gerinnungskaskade. Eine FXII-Defizienz oder Dysfunktionalität ist mit einer verzögerten aktivierten partiellen Thromboplastinzeit (aPTT) assoziiert, nicht aber mit einer erhöhten Blutungsneigung. Dennoch zeigen experimentelle Modelle, dass FXII essentiell für die Bildung arterieller und venöser, Gefäß-verschließender Thrombosen ist. Diese Übersichtsarbeit fasst das aktuelle Wissen über die Struktur von FXII, den Mechanismus der FXII-Kontaktaktivierung und die Bedeutung von FXII für diagnostische Gerinnungstests zusammen.

#### **Schlüsselwörter**

- Kontaktphasesystem
- Blutgerinnung
- Polyphosphat
- Entzündung
- Faktor XII

#### **Proteins of the Plasma Contact System**

The factor XII (FXII)-driven plasma contact system is a proinflammatory and procoagulant plasma protease cascade that is initiated by FXII, in a reaction involving high-molecular-weight kininogen (HK) and plasma prekallikrein (PK; **Fig. 1**).<sup>1-3</sup> Upon

received January 17, 2021 accepted after revision June 11, 2021 binding to negatively charged surfaces, the FXII zymogen is autocatalytically converted to the serine protease FXIIa, which cleaves PK to active plasma kallikrein (PKa). FXIIa initiates the intrinsic pathway of coagulation and leads to the PKa-mediated liberation of the proinflammatory peptide hormone bradykinin

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Fig. 1 Contact system-triggered pathways. The contact system is initiated by factor XII (FXII) activation mediated by contact with anionic surfaces such as polyphosphate (polyP) nanoparticles, mast cell heparin, cell-derived extracellular vesicles, misfolded protein aggregates, and various exogenous surfaces. Activated FXII (FXIIa) drives proinflammatory and procoagulant pathways, such as (1) plasma kallikrein (PK)-stimulated release of bradykinin (BK) and des-Arg9-BK from high-molecular-weight kininogen (HK, kallikrein-kinin system), followed by binding of the released peptide hormones to kinin B2 receptor (B2R) and kinin B1 receptor (B1R); (2) proteolytic cleavage of factor XI (FXI) leading to initiation of the intrinsic coagulation cascade with subsequent thrombin and fibrin formation and clotting; (3) urokinase-type plasminogen activator receptor (uPAR)-mediated activation of plasminogen to the protease plasmin, which degrades formed fibrin clots, resulting in the formation of D-dimers; and (4) activation of the classical complement pathway via the proteases C1r and C1s. While the C1 esterase inhibitor (C1INH) plays an important role in complement inhibition, it is also the main inhibitor of FXIIa and activated PK in vivo.

(BK) from the precursor HK (kallikrein-kinin pathway) in vivo. BK, in turn, is rapidly metabolized to vasoactive and eventually inactive peptides. Furthermore, FXIIa has the capacity to activate the fibrinolytic and complement systems in vitro. However, a possible in vivo relevance of FXII contact activation for these reactions remains to be demonstrated.<sup>4</sup> In contrast to other coagulation factors, deficiency in FXII is not associated with obvious coagulation abnormalities in humans and mice. FXII-deficient individuals are phenotypically normal and do not bleed excessively. Our laboratory originally found that both arterial and venous thrombus formation is defective in FXIIdeficient  $(F12^{-/-})$  mice while their hemostatic capacity is within the normal range.<sup>5</sup> It has also been shown that genetic ablation of F12 in mice leads to impaired venous thrombus formation in an inferior vena cava (IVC) stenosis and femoral vein electrolytic injury model, but not in an IVC stasis model.<sup>6</sup> Even so, challenging the dogma of a coagulation "balance,"  $F12^{-/-}$ animals have a normal hemostatic capacity but are largely protected from thromboembolic diseases such as ischemic stroke,<sup>7</sup> pulmonary embolism,<sup>8</sup> cancer-driven thrombosis,<sup>9</sup> or

sepsis-associated thrombosis.<sup>10</sup> In addition, pharmacological inhibition of FXIIa-driven coagulation provides safe thromboprotection in large animals.<sup>11,12</sup> Peptides, proteins, and smallmolecular-weight inhibitors targeting FXII and FXIIa are discussed in detail by Davoine et al.<sup>13</sup> In addition to coagulation, FXIIa initiates an inflammatory reaction by generation of the peptide hormone BK.<sup>14,15</sup> Further processing of BK by removal of the C-terminal arginine through carboxypeptidase N results in des-Arg9-BK. Binding of BK and des-Arg9-BK to its G-proteincoupled kinin B2 and B1 receptors (B2R and B1R) initiates intracellular Ca<sup>2+</sup>-dependent signaling pathways that induce a plethora of inflammatory processes<sup>16,17</sup> leading to cytoskeleton rearrangement in endothelial<sup>18,19</sup> and epithelial cells.<sup>20</sup> Deficiency in C1 esterase inhibitor (C1INH, the major endogenous inhibitor of FXIIa and PKa) or FXII gain-of-function mutations are associated with a BK-mediated, life-threatening inflammatory disorder, referred to as hereditary angioedema (HAE).<sup>21-23</sup>

### **FXII: Domains and Their Functions**

The plasma protein FXII is secreted by the liver into the circulatory system as an inactive, single-chain enzyme precursor (zymogen). In addition, some blood cells such as neutrophils express FXII<sup>2,24</sup> (https://www.proteinatlas.org/ENSG00000131187-F12/tissue). An N-terminal 19-residue signal sequence mediates secretion of the FXII zymogen. In healthy individuals, the 80 kDa glycoprotein (596 amino acids) circulates in plasma at a concentration of 30 to 35 µg/mL (375 nmol/L).<sup>25</sup> Proteolysis of FXII zymogen occurs at the single peptide bond Arg353-Val354 leading to formation of the active protease FXIIa. Formation of FXIIa proceeds via two principal mechanisms: (1) binding ("contact") to negatively charged surfaces that induce a conformational change and limited enzymatic activity (autoactivation) or (2) by proteases including PKa and plasmin (fluid-phase activation). FXIIa is composed of two chains termed the "heavy chain" (353 residues) and the "light chain" (243 residues). Both chains are linked by an intramolecular disulfide bond spanning cysteine residues 340 and 467. The light chain comprises the catalytic domain with the typical triad of a serine protease (His393, Asp442, Ser544). The heavy chain is composed of individual domains, starting with the N-terminal fibronectin type II (Fib-II) domain, followed by a first epidermal growth factor-like (EGF-I) domain, a fibronectin type I (Fib-I) domain, a second EGF-like (EGF-II) domain, a kringle domain, and a C-terminal proline-rich (PR) region. In particular, the heavy chain mediates contact to other proteins and surfaces with implications for zymogen activation (**Table 1**). A FXII fragment that completely lacks the heavy chain was unable to contact activate.<sup>11</sup> However, the site(s) required for contact activation has/have remained enigmatic and different studies came to discrepant results.

Anti-FXII antibodies that interfere with FXII contact activation have been used to identify residues involved in surface binding. Monoclonal antibodies P5-2-1 and B7C9 identified a region within the Fib-II domain as a FXII surface binding site.<sup>26,27</sup> Challenging a role of residues 1–28 in the Fib-II domain for FXII contact activation, a FXII deletion mutant

Attribute	Fib-II	EGF-I	Fib-I	EGF-II	KR	PR	Catalytic domain
Potential surface-binding site	▶ 26,27	<b>⊮</b> <sup>33</sup>	► <sup>29,30</sup>	► <sup>32,102</sup>	✓ <sup>32,102</sup>		
Putative Zn <sup>2+</sup> -binding site	✓ <sup>35,103</sup>	<b>₩</b> <sup>35</sup>		✓ <sup>35</sup>			
FXI interaction	✓ <sup>28</sup>						
Cell attachment	✓ <sup>104</sup>						
Heparin-binding site			✓ <sup>105</sup>				
Fibrin-binding site			✓ <sup>105</sup>				
Catalytic triad (His393, Asp442, Ser544)							<b>№</b> <sup>106</sup>

 Table 1
 FXII domains/regions and their functions

Abbreviations: Fib-II, fibronectin type II domain; EGF-I, first epidermal growth factor-like domain; Fib-I, fibronectin type I domain; EGF-II, second epidermal growth factor-like domain; KR, kringle domain; PR, proline-rich region.

lacking residues 3–19 was readily contact activatable by dextran sulfate (DXS).<sup>28</sup> The monoclonal antibody B7C9 identified amino acids 134–153 as a part of the Fib-I domain as potential polyanionic surface binding site.<sup>29</sup> Furthermore, anti-FXII antibody KOK5 that recognizes a discontinuous epitope in the Fib-II domain involving residues 30–33, 40–47, and 57–60 inhibits kaolin-triggered FXII contact system activation.<sup>30</sup> However, a 319-amino-acids-deleted FXII mutant, rFXII.lpc, that consists of the light chain and the C-terminal part of the PR region but lacks the entire KOK5 epitope, binds to kaolin and is activated by the anionic surface.<sup>31</sup> A FXII mutant spanning the EGF-II domain, kringle domain, and PR region, as well as the light chain (rFXII-U-like) binds to surfaces and undergoes contact activation.<sup>32</sup> Moreover, a recent study based on recombinant N-terminal FXII deletion mutants indicated that truncated FXII variants lacking the Fib-II domain (FXII  $\Delta$ 1–71) are more prone for autoactivation.<sup>33</sup> This suggests that the Fib-II domain is involved in zymogen contact activation by shielding its activation site. Using various deletion mutants, it was further shown that the Fib-II domain is dispensable for FXII binding to kaolin or polyphosphate (polyP), whereas the EGF-I domain appeared as indispensable.<sup>33</sup> This result is demonstrated in pull-down assays with recombinant FXII variants lacking the EGF-I domain (FXII  $\Delta$ 1–112) that are defective in binding to kaolin and polyP.<sup>33</sup> Together, these studies reveal the complexity in activation of the FXII zymogen and suggest a systematic approach to identify the regions of FXII involved in surface-triggered autoactivation. Functions related to the





kringle domain and PR region are not well characterized. Based on in vitro experiments, these domains influence the conformation of FXII and accordingly the exposure of its cleavage site at Arg353-Val354. A recently described FXII point mutation (Trp268Arg) located in the kringle domain next to the PR region disturbs zymogen quiescence and accelerates FXII activation leading to rash and urticaria<sup>34</sup>; however, the underlying FXIIdependent mechanism remains to be characterized.

## **FXII: Zinc Binding**

The divalent cation zinc  $(Zn^{2+})$  is involved in the binding and activation of FXII on negatively charged surfaces. In particular, Zn<sup>2+</sup> promotes the stabilization of the transition states formed during the conformational change of FXII upon its autocatalytic cleavage. Putative Zn<sup>2+</sup> binding sites within the Fib-II domain (residues His40-His44 and His78-His82) and the EGFlike domains (residues His94-His131 and His174-His176) were described for FXII.<sup>35</sup> Zn<sup>2+</sup> circulates in plasma bound to proteins and is released by activated platelets. In vitro studies have also shown that binding of FXII-stimulated platelets is enhanced in the presence of Zn<sup>2+</sup>.<sup>36</sup> Moreover, Zn<sup>2+</sup> facilitates FXII binding to endothelial and smooth muscle cells promoting mitogenic activity in these cell types.<sup>37</sup> FXII binds urokinase-type plasminogen activator receptor (uPAR) and induces proliferation via *B1* integrin and EGF receptor (EGFR) signaling that leads to phosphorylation of pERK1/2 and Akt.<sup>38</sup> In addition to uPAR, the globular C1q receptor (gC1qR) provides an attachment site for contact system proteins on the cell surface. The interaction of C1q with gC1qR is

known to activate the classical complement cascade<sup>39</sup>; however, gC1gR also binds to FXII and HK and promotes their assembly on cell surfaces.<sup>40</sup> Complex formation with contact system proteins is facilitated by anionic loops of gC1gR. whereas a cell-binding site allows attachment of gC1qR to plasma membrane proteins, such as integrins.<sup>41</sup> Recent structural analysis revealed that the N-terminal Fib-II domain of FXII requires the presence of  $Zn^{2+}$  for interaction with gC1qR.<sup>42</sup> By clustering FXII and HK into a planar ternary complex with a molecular weight of 500 kDa, cell surfacebound gC1gR may enable initiation of the FXII-driven intrinsic coagulation and kallikrein-mediated BK liberation.<sup>42</sup> However, other studies have shown that gC1gR has a mitochondrial targeting sequence sufficient to localize the protein to intracellular compartments, challenging its proposed role as a cell surface receptor for plasma proteins.<sup>43</sup>

# **FXII: Glycosylation**

FXII is a glycoprotein of the  $\beta$ 2 globulin fraction of serum electrophoresis separation. Carbohydrates constitute for 14.2 kDa (17.8%) of the apparent molecular protein mass of 80 kDa. In human FXII, two *N*- and seven putative *O*-glycosylation sites have been identified (**-Table 2**). Most of the glycosylation sites are located within the PR region. Glycosylation is crucial for the functionality and the physical properties of FXII. Consistent with this, a nonglycosylated FXII variant expressed in *Escherichia coli* has very low or no solubility (unpublished observation of the authors). While glycosylation is critical for solubility of the plasma protein,

FXII domain/ region	Type of glycosylation	Amino acid	Amino acid position	Reference	Related findings	Reference
EGF-I	O-linked	Thr	90	107		
Kringle	<i>N</i> -linked	Asn	230	102,108	Asn230Lys mutation (recom- binant model, <i>Schneider</i> 2 (S2) cells): lack of glycosyla- tion results in increased in- tracellular FXII levels and defective secretion	109
Proline-rich	O-linked	Thr	280	102		
	O-linked	Thr	286	102		
	O-linked	Ser	289	102		
	O-linked	Thr	309	102	Thr309Lys/Arg mutation: loss of glycosylation leads to increased FXII activity in HAE- FXII patient plasma	45
	O-linked	Thr	310	102		
	O-linked	Thr	318	102		
Catalytic domain	<i>N</i> -linked	Asn	414	108,110	Hypoglycosylated type of FXII in plasma of PMM2-CDG patients: FXII contact activa- tion is unaffected	109

Table 2 Glycosylation sites of FXII

Abbreviation: EGF-I, first epidermal growth factor-like domain.

carbohydrates also regulate FXII contact activation. For example, contact activation of a hypoglycosylated FXII variant with a missense mutation at position 309 (Thr309Lys/Arg; identical to position 328 counting the 19 amino acid signal peptide) is largely accelerated.<sup>44</sup> Lack of a single O-linked FXII glycosylation leads to a gain-of-autoactivation.<sup>45</sup> Furthermore, the mutation produces an additional site for activation of the zymogen by plasmin.<sup>46</sup> Synergistically, gain-of-contact activation and an extra cleavage site lead to excessive production of BK. BK-signaling increases microvascular permeability leading to the inherited swelling disease HAE. Three distinct types of HAE can be differentiated: mutations in the gene SERPING1 encoding for C1INH are causative for HAE type I and HAE type II. Patients suffering from these forms of HAE exhibit a decreased plasma concentration and impaired functionality of C1INH, respectively. In contrast, the third type represents HAE with normal C1INH (HAE-nC1INH) associated with mutations of genes other than SERPING1, such as KNG1,<sup>47</sup> ANGPT1,<sup>48</sup> PLG,<sup>49</sup> or F12.<sup>50</sup> A molecular aberration in F12 leads to the aforementioned Thr309Lys/Arg FXII mutation and causes HAE-FXII due to the increased susceptibility of FXII for contact activation.<sup>45</sup>

#### **FXII:** Activation

#### **Endogenous Activators**

Over the years, an array of physiological structures with anionic surfaces have been identified as putative FXII contact activators. Here, we focus on some of them, including mast cell heparin, misfolded protein aggregates, polyP, and cellderived extracellular vesicles. A complete overview on FXII contact activators can be found here.<sup>1</sup>

Heparin, a polysaccharide with a high degree of sulfation and acetylation, is released from mast cells stimulated by IgE/antigen. Mast cell heparin initiates FXII contact activation in human plasma and in mice.<sup>23</sup> Subsequently, BK is released by the kallikrein–kinin system,<sup>15</sup> inducing vasodilation and an increase in permeability with implications for anaphylaxis<sup>51</sup> and edema.<sup>23</sup> In contrast, deficiency of FXII or B2R that is associated with defective BK signaling interferes with mast cell heparin-driven acute swelling events in vivo.<sup>23</sup>

The amyloid  $\beta$  (A $\beta$ ) peptide is one of the pathological hallmarks of Alzheimer's disease (AD) and, in its aggregated form, has the capacity to initiate FXII contact activation. In particular, the A $\beta$ 42 isoform induces thrombin generation via the intrinsic coagulation cascade in a FXIIa-mediated manner<sup>52</sup> and induces inflammatory reactions by the activated kallikrein–kinin system. Elevated levels of FXIIa are found in the plasma of AD patients,<sup>53</sup> consistent with a role of the contact system in procoagulant and proinflammatory states associated with AD.

#### Polyphosphate

It is known for decades that activated platelets promote coagulation in a FXII-dependent manner, <sup>54–59</sup> but the platelet-derived FXII activator had remained unknown. Platelets store high concentrations of the inorganic polymer polyP in their dense granules.<sup>60</sup> PolyP is a linear polymer composed of orthophosphate [P], monomers that are linked by energyrich phosphoanhydride bonds. PolyP complexed with calcium ions (Ca<sup>2+</sup>) is packed in dense granules in high concentrations (up to the molar range) leading to characteristic dark spots in transmission electron microscopy images. Activated platelet-derived polyP potently initiates FXII contact activation<sup>8</sup> providing the long-sought link of primary and secondary hemostasis.<sup>61</sup> PolyP is instable in plasma and is degraded by endogenous phosphatases.<sup>62</sup> Targeting polyP using synthetic and recombinant binding proteins or exopolyphosphatases, enzymes that degrade polyP in bacteria and yeast, interferes with the activity of the polymer to induce FXII contact activation.<sup>63</sup> Only a small portion of platelet polyP (<5%) is released into the cell supernatant upon activation,<sup>63</sup> but the vast majority remains attached to the plasma membrane, indicating that the polymer operates on cell surfaces. In contrast to synthetic polyP, naturally occurring polymers are bound to divalent metal cations such as  $Ca^{2+}$  (and possibly others, e.g.,  $Zn^{2+}$ ). The resulting aggregate formation leads to insoluble polyP nanoparticles that are retained on the platelet membrane.<sup>64</sup> Intravital microscopy has visualized polyP nanoparticles on the surfaces of activated platelets and within platelet-rich thrombi confirming that the majority of platelet polyP is retained on the plasma membrane.<sup>65</sup>

The insoluble  $Ca^{2+}$  and  $Zn^{2+}$ -rich polyP nanoparticles found in nature challenge the hypothesis that size of individual polymers would determine polyP function in coagulation. While chain length of synthetic polyP determines its activity on coagulation reactions in plasma,<sup>66</sup> natural platelet polyP forms insoluble Ca<sup>2+</sup>-rich nanoparticles. These particles function in coagulation reactions independently of the chain length of the individual polyP molecule.<sup>65</sup> Furthermore, these particulate polyP form triggers FXII activation on plasma membranes of platelets, megakaryocytes, various cancer cells, and exosomes/microparticles derived from these cells, with implications for thrombosis in murine models.<sup>9</sup> In contrast to insoluble particulate polyP, a potential in vivo function of soluble polyP remains to be shown. Notably, soluble polyP may have anticoagulant effects since Ca<sup>2+</sup>-free synthetic short-chain polyP acts as a chelator for Ca<sup>2+</sup> ions in plasma.<sup>66</sup>

Platelets release small amounts of short chain polyP (around 80 monomers) that is soluble and found in the supernatant. However, and similar to other cells the majority of platelet polyP comprises long chain insoluble polymers with a chain length  $> 200 [P]_i$  subunits. Methods to isolate polvP from cells and tissues have been established. The phenol/chloroform extraction method (Werner's protocol<sup>67</sup>) selects for water soluble (short chain) polyP, while anion exchanger is based on purification of both soluble (short chain) and insoluble (long chain) polyP.68 Despite that polyP is found in every cell in nature, the biosynthesis of the polymer is a topic of ongoing research. So far, mainly prokaryotes but also yeast have served as model organisms for the analysis of enzymes that synthesize polyP with different chain lengths.<sup>68</sup> In yeast, intracellular levels of polyP and inositol pyrophosphate are interdependent from

each other and inositol pyrophosphate modulates the cellular [P]; influx.<sup>69</sup> The concentrations of both molecular species are regulated by inositol hexakisphosphate kinase (IP6K1) activity. In Saccharomyces cerevisiae and mice, genetic ablation of Ip6k1 severely reduces polyP and inositol pyrophosphate levels.<sup>70</sup> While reduced inositol pyrophosphate levels do not significantly affect secondary hemostasis, decreased platelet polyP impairs activation of the coagulation cascade in a FXII-dependent manner.<sup>71</sup> Consequently, IP6K1 deficiency protects genetically modified mice from lethal venous thromboembolic events. The xenotropic and polytropic receptor 1 (XPR1) that was originally desired as [P]<sub>i</sub> exporter represents another polyP-regulating protein. Genetic and pharmacologic targeting of XPR1 interferes with [P]; export and results in polyP accumulation in platelets.<sup>72</sup> Accordingly, surface exposure of polyP from stimulated XPR1-deficient platelets is increased. Excess platelet polyP has no effect on hemostasis but accelerates arterial and venous thrombosis in a FXII-dependent manner. Therefore, modulating proteins that regulate polyP content in platelets may be useful for the prevention of thrombotic disorders.

#### **Cell-Derived Extracellular Vesicles**

Extracellular vesicles (EV) modulate blood coagulation under physiological and pathological conditions. Originally, platelet-derived EV termed "microparticles" have been shown to initiate FXII contact activation, while red blood cell-derived EV were inactive in producing FXIIa.<sup>73</sup> Flow cytometric and biochemical analyses with EV isolated from platelet concentrates confirmed that EV support thrombin generation in an FXII-dependent manner. However, in that specific study, red blood cells were also found to contribute to EV-driven FXIIa production,<sup>74</sup> suggesting that distinct populations or agedependent effects on the coagulation system exist in red blood cells. Red blood cell-derived EV participate in activation of the intrinsic coagulation pathway by two mechanisms: (1) the classical contact activation and (2) a FXIIindependent pathway involving PK activation followed by direct stimulation of factor IX (FIX) by a yet unknown protease.<sup>75</sup> Recently, and in line with the previous study, PKa-mediated FIX activation was confirmed in plasma ex vivo.<sup>76</sup> The clinical consequence of red blood cell-triggered coagulation may explain the procoagulant state in patients after blood transfusions.

Biogenesis of EV is accompanied by the translocation of the phospholipid phosphatidylserine (PS) from the inner to the outer membrane leaflet.<sup>77</sup> In addition, exposure of PS on the cell surface is a hallmark of programmed cell death. Apoptotic cells are procoagulant by presenting PS as an attachment site for FXII.<sup>78</sup> A role of phospholipids in FXII-triggered contact activation is also known from circulating triglyceride-rich particles. Thromboelastography and thrombin generation assays revealed that very low density lipoproteins mediate FXII contact activation in a phosphatidylethanolamine- but not PS-dependent manner.<sup>79</sup> In conclusion, further studies are required to analyze, whether phospholipids, especially PS, have a supportive role in FXII-driven coagulation or

whether they are capable to directly induce FXII contact activation.

#### **Exogenous Activators**

The white clay material kaolin mainly contains kaolinite, a hydrated aluminum silicate. Similar to Ca<sup>2+</sup>-polyP, Ca<sup>2+</sup>kaolin provides a negatively charged surface for FXII binding and subsequent contact activation.<sup>80</sup> Due to these procoagulant activities, hemostatic wound dressings (QuikClot Combat Gauze) are coated with kaolin to rapidly control trauma-induced bleeding. However, long-term exposure of the injured tissue to these hemostatic dressings increases the thromboembolic risk, as kaolin can enter the circulation and trigger clot formation.<sup>81</sup> Wounds rarely remain clean and are more often contaminated with soil, especially in terrestrial vertebrates. Soil is predominantly composed of silicate minerals and induces activation of the intrinsic coagulation cascade in a FXII-dependent manner.<sup>82</sup> Consistent with previous studies showing that birds and some marine mammals are deficient in FXII,<sup>83,84</sup> soil-stimulated clotting was defective in dolphin or chicken blood.<sup>82</sup> In a murine bleeding model, treatment of an injured vessel with a polyacrylic acidcoated filter paper reduced underlying blood loss in a FXIIdependent manner, indicating that the polymer functions as a contact activator.85

In addition to kaolin and other silica-rich materials, glass, sulfatides, and ellagic acid (EA) also represent artificial surfaces that initiate FXII contact activation.<sup>86,87</sup> EA is a polyphenolic compound associated with antiproliferative and antioxidant effects. Similarly with kaolin and polyP, EA forms complexes with divalent metal ions<sup>88</sup> and only these EA particles, but not soluble free EA molecules, are capable to induce FXII contact activation.<sup>88</sup> Administration of an infusion containing EA caused thrombosis in patients by direct activation of FXII or platelet stimulation.<sup>89</sup>

A well-known FXII contact activator is the sulfated polysaccharide DXS. High-molecular-weight DXS (500 kDa) stimulates FXII contact activation, whereas short-chain DXS (5 kDa) is inactive for zymogen activation.<sup>90</sup> Challenging pigs with DXS led to a BK-mediated drop in blood pressure<sup>91</sup> but did not induce thrombosis. This suggests that DXS specifically triggers the kallikrein-kinin system leading to inflammation but is inactive for driving coagulation. Therefore, injection of the polymer is useful to analyze BK-mediated edema independently on coagulation in mouse models.<sup>92</sup> The synthetic polysaccharide oversulfated chondroitin sulfate (OSCS) acts similarly with high-molecularweight DXS. OSCS is a potent FXII contact activator. Infusion of heparin contaminated with OSCS led to hypotonic shock<sup>93</sup> with fatal outcome due to BK-mediated hypersensitivity reactions.94

The structurally related glycosaminoglycan fucosylated chondroitin sulfate (FCS) also activates the contact system in human plasma. Based on carbohydrate–protein interaction studies, PK binds readily to FCS in the presence of the other contact system proteins, leading to the hypothesis that FXIIa is generated mainly by PKa-mediated fluid-phase activation.<sup>95</sup>

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Table 3         F12         gene mutations         associated with

Case no.	Exon/Intron	Genetic variation	Comments	Protein	Condition	c4 genotype	FXII:C	FXII:Ag	Reference
1	Exon 1	c13C > T	5' UTR	1	Heterozygous FXII deficiency	C/T	22%	1	112
2	Exon 1	c13C > T	5' UTR	1	Compound heterozygous FXII	I	< 1%	< 1%	113
		c8C > G	5' UTR	I	deficiency; CRM-negative				
3	Exon 1	c8C > G	5' UTR	1	Homozygous FXII deficiency	т/т	< 1%	I	112
4	Exon 1	c8C > G	5' UTR	I	Homozygous FXII deficiency;	I	< 1%	1%	114,115
	Intron B	c.116-224C > T	Tagl restriction site	1	CRM negative				
ъ	Exon 1	c8C > G	5' UTR	1	Compound heterozygous FXII	1	< 1%	< 1%	113
	Exon 13	c.1558T > C	Missense mutation	p.Gln520* (Gln501*)	deficiency; CRM-negative				
6	Exon 1	c8C > G	5' UTR	1	Compound heterozygous FXII	I	< 1%	< 1%	113
	Exon 14	c.1697C > T	Missense mutation	p.Pro566Leu (Pro547Leu)	deficiency; CRM-negative				
7	Exon 3	c.158A > G	Missense mutation	p.Tyr53Cys (Tyr34Cys)	FXII Tenri, homozygous FXII deficiency; CRM-negative	I	3%	3%	116
8	Exon 4	c.218G > C	Missense mutation	p.Cys73Ser (Cys54Ser)	Heterozygous FXII deficiency	т/т	1%	I	117,118
6	Exon 4	c.249delG	Frameshift mutation	p.Gln83Hisfs*12 (Gln64Hisfs*12)	Compound heterozygous FXII	C/T	< 0.5%	I	119
	Exon 6	c.405C > A	Nonsense mutation	p.Cys135* (Cys116*)	deficiency				
10	Exon 4	c.251G > C	Missense mutation	p.Arg84Pro (Arg65Pro)	Homozygous FXII deficiency	τ/τ	< 10%	<17%	120
11	Exon 5	c.303_304delCA	Frameshift mutation	p.His101Glufs*36 (His82Glufs*36)	Heterozygous carrier	C/T	39%	41.6%	121
12	Exon 5	c.303_304delCA	Frameshift mutation	p.His101Glufs*36 (His82Glufs*36)	Homozygous FXII deficiency	τ/τ	%0	1.2%	121
13	Exon 6	c.405C > A	Nonsense mutation	p.Cys135* (Cys116*)	Homozygous FXII deficiency	т/т	<0.5%	I	119,122
14	Exon 6	c.425G > C	Missense mutation	p.Arg142Pro (Arg123Pro)	Homozygous FXII deficiency; CRM-negative	т/т	<1%	<10%	123
15	Exon 7	c.583delC	Frameshift mutation	p.His195Thr*55 (His176Thr*55)	Heterozygous carrier	C/T	53.4%	1	118
16	Exon 7	c.583delC	Frameshift mutation	p.His195Thr*55 (His176Thr*55)	Compound heterozygous FXII	т/т	<1%	I	118
	Exon 10	c.1092_1093insC	Frameshift mutation	p.Lys365fs*68 (Lys346fs*68)	deficiency				
17	Exon 8	c.721T > G	Missense mutation	p.Trp241Gly (Trp222Gly)	Heterozygous carrier	I	40%	55%	124
18	Exon 8	c.721T > G	Missense mutation	p.Trp241Gly (Trp222Gly)	Compound heterozygous FXII	I	<3%	<10%	124
	Exon 12	c.1396C > A	Missense mutation	p.Arg466Ser (Arg447Ser)	deficiency; CRM-negative				
19	Exon 8	c.776G > A	Missense mutation	p. Gly259Glu (Gly240Glu)	Homozygous FXII deficiency; CRM-negative	c/c	0.6%	<1%	125
20	Exon 8	c.799C > G	Missense mutation	p.Arg267Gly (Arg248Gly)	Homozygous FXII deficiency; CRM-negative	c/c	1.5%	<1%	125
21	Exon 9	c.809_811delACA	Deletion mutation	p.Asn271del (Asn252del)	Compound heterozygous FXII	I	2.0%	5.2%	126
	Exon 10	c.1078G > A	Missense mutation	p.Gly360Arg (Gly341Arg)	deficiency				
22	Exon 9	c.856_864delAGCTGGGAG	Frameshift mutation	p.Ser286_Glu288 (Ser267_Glu269)	Heterozygous FXII deficiency; CRM-negative	т/т	<1%	<1%	127
									(Continued)

Case no.	Exon/Intron	Genetic variation	Comments	Protein	Condition	c4 genotype	FXII:C	FXII:Ag	Reference
23	Exon 10	c.1027G > C	Missense mutation	p.Ala343Pro (Ala324Pro)	Heterozygous carrier	т/т	29%	30%	128
24	Exon 10	c.1027G > C	Missense mutation	p.Ala343Pro (Ala324Pro)	Compound heterozygous FXII	T/C	35%	81%	128
	Exon 13	c.1649G > A	Missense mutation	p.Gly550Glu (Gly531Glu)	deficiency; CRM-negative (Ala324Pro) and CRM-positive (Gly531Glu)				
25	Exon 10	c.1078G > A	Missense mutation	p.Gly360Arg (Gly341Arg)	Homozygous FXII deficiency	т/т	12%	10%	129
26	Exon 10	c.1078G > A	Missense mutation	p.Gly360Arg (Gly341Arg)	Heterozygous carrier	C/T	35%	38%	129
27	Exon 10	c.1078G > A	Missense mutation	p.Gly360Arg (Gly341Arg)	Compound heterozygous FXII	1	2.0%	1.0%	126
	Exon 13	c.1561G > A	Missense mutation	p.Glu521Lys (Glu502Lys)	deficiency				
28	Exon 10	c.1092_1093insC	Frameshift mutation	p.Lys365fs*68 (Lys346fs*68)	Heterozygous carrier	т/т	17.1%	1	118
29	Exon 10	c.1093_1094insC	Frameshift mutation	p.Lys365GInfs*69 (Lys346GInfs*69)	Compound heterozygous FXII	C/T	<0.5%	1	119,122
	Exon 14	c.1744G > A	Missense mutation	p.Gly582Ser (Gly563Ser)	deficiency				
30	Exon 10	c.1095G > C	Missense mutation	p.Lys365Asn (Lys346Asn)	FXII Ofunato, CRM-reduced FXII deficiency	т/т	5%	4.5%	130
31	Exon 10	c.1115G > C	Missense mutation	p.Arg372Pro (Arg353Pro)	FXII Locarno, CRM-positive FXII deficiency	I	<1%	46%	131,132
32	Exon 10	c.1231G > A	Missense mutation	p.Ala411Thr (Ala392Thr)	Factor XII Shizuoka, homozy- gous FXII deficiency; CRM- negative	т/т	<3%	<10%	133
33	Exon 10	c.1240C > A	Missense mutation	p.Leu414Met (Leu395Met)	Heterozygous carrier	I	<28%	34%	115
34	Exon 10	c.1240C > A	Missense mutation	p.Leu414Met (Leu395Met)	Compound heterozygous FXII	1	<5%	5%	115
	Intron M	c.1681-1G > A	Acceptor splice site mutation	1	deficiency; CRM-negative				
35	Exon 11	c.1318C > A	Missense mutation	p.Gln440Lys (Gln421Lys)	Heterozygous carrier; CRM- negative	C/T	23%	28%	123
36	Exon 12	c.1515G > C	Missense mutation	p.Trp505Cys (Trp486Cys)	FXII Mie-1, homozygous FXII deficiency	т/т	<5%	<5%	134
37	Exon 13	c.1561G > A	Missense mutation	p.Glu521Lys (Glu502Lys)	Homozygous FXII deficiency; CRM-negative	C/C	4%	3.8%	125
38	Exon 13	c.1561G > A	Missense mutation	p.Glu521Lys (Glu502Lys)	Heterozygous carrier	c/c	34%	33%	135
39	Exon 13	c.1561G > A	Missense Mutation	p.Glu521Lys (Glu502Lys)	Compound heterozygous FXII	C/T	4%	5%	135
		c.1637T > C	Missense mutation	p.Met546Thr (Met527Thr)	deticiency; CRM-negative				
40	Exon 13	c.1583C > T	Missense mutation	p.Ser528Phe (Ser509Phe)	Heterozygous carrier	т/т	29%	I	122
41	Exon 13	c.1583C > T	Missense mutation	p.Ser528Phe (Ser509Phe)	Compound heterozygous FXII	т/т	5%	I	119,122
	Exon 14	c.1744G > A	Missense mutation	p.Gly582Ser (Gly563Ser)	deficiency				
42	Exon 13	c.1637T > C	Missense mutation	p.Met546Thr (Met527Thr)	Heterozygous carrier	C/T	30%	32%	135

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Case no.	Exon/Intron	Genetic variation	Comments	Protein	Condition	c4 genotype	FXII:C	FXII:Ag	Reference
43	Exon 13	c.1669G > A	Missense mutation	p.Asp557Asn (Asp538Asn)	Heterozygous FXII deficiency	I	5%	6.8%	136
44	Intron M	c.1681–1G > A	Acceptor splice site mutation	1	Heterozygous carrier	I	<26%	<40%	115
45	Intron M	c.1681–1G > A	Acceptor splice site mutation	I	Homozygous FXII deficiency; CRM-negative	1	<1%	<1%	115,137
46	Exon 14	c.1681G > A	Missense mutation	p.Gly561Ser (Gly542Ser)	Homozygous FXII deficiency	I	<1%	<1%	126
47	Exon 14	c.1765G > C	Missense mutation	p.Gly589Arg (Gly570Arg)	Heterozygous carrier; CRM- positive	1	10%	74%	115
48	Exon 14	c.1768T > A	Missense mutation	p.Cys590Ser (Cys571Ser)	FXII Washington, CRM-positive FXII deficiency	1	<1%	80%	138
49	Exon 14	c.1744G > A	Missense mutation	p.Gly582Ser (Gly563Ser)	Heterozygous carrier	T/T	25%	I	122

Abbreviations: CRM, cross-reacting material; FXII:C, factor XII activity; FXII:Ag, factor XII antigen levels.

Notes: Letter prefixes indicate the type of reference sequence: "c."—coding DNA reference sequence (NM\_000505.4), "p."—protein reference sequence (NP\_000466.2). The protein reference sequence contains an N-terminal signal peptide with a length of 19 amino acids. Changes in the amino acid sequence of the mature FXII protein are described in (parentheses). The c.-4 genotype indicates a C/T polymorphism localized in the 5' untranslated region (5' UTR) at nucleotide position four upstream of the translation initiation codon ATG. Nucleotide as well as amino acid changes are depicted as follows: " - "-substitution. "\*"-translation termination codon. 'del"—deletion, "ins"—insertion, "fs"—frameshift,

All of the described endogenous and exogenous FXII contact activators have a net negative surface charge, which appears to be necessary for binding/activation of FXII. Vice versa, the positively charged polyethylenimine and the inert polymer Teflon AF failed to induce FXII contact activation human plasma.<sup>96</sup>

# Relevance of FXII for Diagnostic Coagulation Tests

FXII contact activation is the mechanistic basis for one of the most commonly performed diagnostic coagulation screening tests, the activated partial thromboplastin time (aPTT). aPTT assays are performed more than 5 billion times annually and are used for (1) preoperative screening; (2) monitoring of intravenous application of heparin; (3) detection of lupus anticoagulants/antiphospholipid syndrome; and (4) identification of deficiency in the contact system proteins including FXII, PK, and HK and the coagulation factors FXI, FIX, and FVIII. Kaolin, micronized silica, or EA is commonly used to trigger FXII-dependent clotting in aPTT assays. Initially, citrated plasma is incubated with artificial surfaces for up to 90 seconds. Within that time a substantial amount of FXII zymogen undergoes conversion to FXIIa that subsequently activates factor XI (FXI) to FXIa. Upon recalcification, the coagulation cascade proceeds and eventually forms fibrin. Accordingly, aPTT represents the time that elapses from recalcification to the formation of a fibrin clot. FXII-deficient plasma has a prolonged/abnormal aPTT, as clotting is impaired in vitro. Notably, extending the incubation time with artificial surfaces normalizes aPTT in PK-deficient plasma due to surface-mediated FXII contact activation that compensates for the absence of PKa-mediated fluid-phase FXII activation.<sup>97</sup> aPTT assays could be discussed as a tool for diagnosing C1INH-HAE, as in one study, approximately 73% of HAE patients with C1INH deficiency had a shortened aPTT compared with HAE patients with functional C1INH.<sup>98</sup>

On the basis of abnormal prolonged aPTT results and sequencing analysis, several mutations in the *F12* gene have been identified that were not associated with a history of bleeding events or pathological hemostatic capacity (**-Table 3 -Fig. 2**). This may be one reason why congenital FXII deficiency, as an autosomal recessive inherited disorder, tends to be diagnosed incidentally during routine coagulation testing, e.g., prior to surgery. Most of the reported *F12* mutations result in a quantitative defect (cross-reacting material [CRM]-negative FXII deficiency) represented by a markedly reduced FXII activity (FXII:C) and antigen levels (FXII:Ag). However, a few genetic variations within the *F12* gene are causative for a qualitative defect (CRM-positive FXII deficiency) indicated by a decreased FXII:C but nearly normal FXII:Ag.

In addition to the *F12* mutations described in **Table 3**, a polymorphism in the 5'-untranslated region of *F12* at nucleotide position four upstream of the translation initiation codon ATG (c.-4C > T, referred to as 46 C/T)<sup>99</sup> has additional implications for FXII:C and FXII:Ag. Transcriptional and translational analyses showed that this 46 T allele is associated with decreased translational efficiency compared with 46 C, resulting in low FXII:C and FXII:Ag.<sup>99</sup>

Reduced FXII levels may also be associated with the transfusion of blood cell or platelet concentrates.<sup>100</sup> Platelet activation and subsequent microparticle release occurs upon extended storage times,<sup>101</sup> which then drives FXII activation by exposure to PS and platelet membrane-bound polyP. Thus, reduced FXII levels appear as a consequence of zymogen "consumption" in patients receiving platelet concentrates.

# Conclusion

Because of its selective role in thrombosis-sparing hemostasis, FXII has gained considerable interest as a target for safe anticoagulation over the last decades. In addition, FXII is a promising target for interference with proinflammatory BKmediated responses. Contact activation of FXII is still incompletely understood, and identification of the "FXII contact activation site" will represent a major step forward. Detailed insight into the mechanism of FXII activation will help develop specific FXII contact inhibitors for potential clinical and diagnostic applications.

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#### **Conflict of Interest**

The authors declare that they have no conflict of interest.

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