Título: COAGULOPATHY IN CONTACT: New approaches to investigate FXI deficient coagulopathy

PseudóniMo: COAGULOPATHY IN CONTACT

Resumen

La deficiencia del FXI es una coagulopatía congénita denominada hemofilia C. Un desorden raro pero subestimado por su dificultad diagnóstica y escasa clínica hemorrágica. Pertenece a la ruta de contacto de la cascada de la coagulación aunque también es nexo con la vía extrínseca ya que su activación por la trombina media la ruta de amplificación. Nos encontramos ante un renacimiento del interés por la ruta clásica de contacto del sistema hemostático. Se trata de serín proteasas localizadas en una encrucijada de tres sistemas claves: coagulación, inflamación e inmunidad innata, y con aparente escaso impacto clínico/biológico. Recientemente se han descubierto nuevas funciones para el FXI más allá de las clásicas, y se han abierto nuevas posibilidades de implicación clínica y terapéutica. Pero todavía quedan muchas incógnitas por resolver en esta ruta. El objetivo pretende ampliar el conocimiento del FXI, concretamente: 1) identificar y caracterizar nuevas alteraciones moleculares en *F11*, analizando su impacto funcional y clínico; 2) estudiar nuevos papeles del FXI en distintos contextos: a) cirugía cardiaca valvular; b) COVID-19; c) TRALI; d) anticuerpos antifosfolípido; 3) evaluar la seguridad de los nuevos fármacos dirigidos contra la el FXI; 4) desarrollar nuevos sistemas de análisis. El proyecto se sustenta en un equipo multidisciplinar que ha recogido una de las cohortes más importantes de pacientes con alteraciones moleculares en el *F11* y serán estudiados con diferente metodología molecular y bioquímica ya disponible en nuestro grupo, y en estrecha colaboración con grupos nacionales e internacionales de excelencia en el campo. Presentamos un proyecto viable, aplicando tecnologías de vanguardia como la secuenciación por nanoporos, o desarrollando nuevos sistemas de análisis de las formas activadas. Pretendemos sustentar que

la deficiencia de FXI se encuentra subestimada, identificar nuevas alteraciones moleculares, mecanismos causantes de deficiencia, analizar la expresión hepática de estas proteínas, y demostrar el impacto de este sistema en diferentes patologías. Nuestros resultados aclararán la utilidad clínica del estudio de FXI, así como las posibilidades terapéuticas de la inhibición de esta ruta.

Summary

FXI deficiency is a congenital coagulopathy called hemophilia C. A rare but underestimated disorder due to its diagnostic difficulty and limited hemorrhagic symptoms. It belongs to the contact pathway of the coagulation cascade, although it is also linked to the extrinsic pathway since its activation by thrombin mediates the amplification pathway. We are facing a revival of interest in the classical contact route of the hemostatic system. The classic contact pathway of the coagulation is actually located in a crossroad of three key systems involved in the human homeostasis: coagulation, inflammation and innate immunity. However, the clinical impact of this system was little explored until the last 10 years. Animal models have shown new information on the contact proteases that has increased the interest on the study of these proteins. Novel functions are being discovered for FXI beyond their classical hemostatic and inflammatory roles and the therapeutic application of this element does not stop growing. However, there are still many challenges in this field. Thus, increasing efforts are required to unravel the contact pathway expanding our knowledge of FXI. Our specific objectives are: 1) to identify and characterize new molecular alterations in *F11*, evaluating their functional and clinical impact; 2) to study new roles for FXI in different clinical frameworks: a) cardiac valve replacement; b) COVID-19; c) TRALI; d) antiphospholipid antibodies; 3) to evaluate the safety for new antithrombotic drugs targeting the contact system; 4) to develop new systems for analyzing the contact pathway. The project is supported by a multidisciplinary team that has recruited one

of the largest cohorts of patients with molecular abnormalities in *F11* genes. In close collaboration with groups of excellence in the field, we are presenting a viable and original project by using vanguard technology such as nanopore sequencing and developing new methods to study this system, particularly the active forms. We aim to demonstrate that FXI deficiency is underestimated, to identify new genetic variants or mechanisms with pathogenic consequences, clarify if this system is affected and play a role in the development and evolution of different candidate disorders, and explore new possibilities and safety on the inhibition of this protein in different clinical settings.

Memory

The contact system received this name because they required contact with artificial, negatively charged surfaces for zymogen activation *in vitro*. Importantly, the proteins belonging to the contact system constitute part of three key the organism's homeostatic systems: inflammation coagulation and innate immunity (Figure 1) (1). Thus, the activation of the contact system have been implicated in various types of human disease including septicemia and endotoxemia, acute respiratory distress syndrome (ARDS), disseminated intravascular coagulopathy (DIC), typhoid fever, Rocky Mountain spotted fever, Crohn's disease, transfusion reactions, renal allograft rejection, nephrotic syndrome, hereditary angioedema, and in extracorporeal circulation (2). Unfortunately, the role of the contact system has only been clearly defined in a few disorders, and there are many questions related to the pathophysiological role of these proteins. The best example is the role of the contact system in hemostasis; while deficiency of any protein of the contact system prolongs coagulation times triggered by silica or ellagic acid (mainly aPTT), patients or animal models lacking these proteins have null or minor bleeding consequences (3). In the last 10 years, our understanding of the contact system, particularly its biology and pathophysiology has greatly increased revealing new surprising information. Using gene-modified animal models, inhibitors and epidemiological studies of patients with deficiency of contact system proteins, revealed that these molecules may be excellent targets for new antithrombotic treatments that have less side effects than classical ones (4). Thus, we are assisting to the renaissance of the interest in the contact system, and new approaches are required to get new information concerning the proteins of this pathway, to evaluate new situations or conditions where these elements might play a clinical impact, to study new therapeutic options related to this system, and to develop new methods to study this system.

This system consists of three serine proteinases: FXII, FXI and plasma prekallikrein (PK), and the nonenzymatic cofactor high molecular weight kininogen (HK). FXII is the first protein of the

contact system that undergoes conformational changes to become autoactivated once bound to negatively changed surfaces. The activated FXII (FXIIa) may proteolyze and thus activate both PK and FXI, in a proteolytic cascade that also requires strong inhibitors to become controlled (Figure 1) (2).

Deficiency of FXI has been considered as rare diseases (FXI Deficiency: ORPHA:329), but there are increasing evidences that this coagulopathy might be underestimated, because diagnostic methods are quite unspecific and insensitive (1,5). Indeed, our group identified a large cohort of patients with FXI deficiency in a Spanish town by analyzing cases with prolonged aPTT (6), demonstrating that the most recurrent mutation, p.Cys38Arg has an ancient origin (5400 years) and a wide distribution in Europe (7,8).

In *F11* the number of genetic variants (N= 191) are most of them associated to FXI deficiency (http://www.factorxi.org/). Moreover, the mechanisms involved in the deficiency of this protein is not fully known, and the clinical impact is largely unknown. Therefore, the discovery of new variants affecting this molecule is required to identify new functional residues or domains, as well as to identify new mechanisms involved in its deficiency, impaired function, increased activation capacity. It is also possible to find new variants with new functions. These studies may also have therapeutic applications, as they may help the development of new antithrombotic drugs, or new drugs for other disorders.

The number of disorders in which the contact system may be involved is continuously growing. Originally, most studies were focused to find any relationship of the contact proteins with bleeding disorders, probably greatly influenced by the prolongation of aPTT observed in patients with severe deficiency of these proteins. Indeed, FXI deficiency was called C hemophilia. But increasing evidences are supporting a minor risk of bleeding in these patients, there is no relationship between the bleeding tendency and the FXI protein levels or the *F11* genetic variant, so it is plausible that the combination with other factors may trigger bleeding in these patients

(9). In contrast, animal models and epidemiological data support that deficiency or inhibition of FXI significantly protects against thrombotic disorders more efficiently than current antithrombotic drugs with minor bleeding risk (10–13). Thus, different strategies targeting this molecule is being developed with therapeutic objective, some of them already evaluated in clinical trials with excellent perspectives (14,15). However, it would be interesting to show further evidence on the safety of treatments targeting FXI. This must include evaluating potential additive or synergic effects of other treatments affecting the hemostatic or inflammatory system, which according to our knowledge have not been done.

Moreover, the strategic position of the contact system, playing a role in coagulation, inflammation and innate immunity, strongly support that this system might be involved in many other diseases. Indeed, it is also possible that contact proteins might have new functions not characterized. Therefore, it is mandatory to evaluate the contact system in other diseases or situations where it may be involved. In this framework, patients requiring medical devices that might activate the contact system, such as vascular catheters, cardiac valves, as well as patients exposed to extracorporeal membrane oxygenation systems or dialysis are probably the first candidates to be evaluated. There are evidences of the potential benefit of targeting the contact system in these situations but these studies have only been done in animal models (16). Thus, clinical studies using samples of patients exposed to artificial surfaces are required. Moreover, the role of the contact system in different disorders should be studied, particularly in those involving inflammation, coagulation and innate immunity. Probably the best example and unfortunately of acute importance is COVID-19 (17,18). Some studies suggested the importance of the contact pathway in this disease. It is increasing the interest of knowing the impact and mechanisms on the hemostasis system and to discover new biomarkers and pharmacological targets to avoid thrombotic complications. In this context this molecule such as FXI is a good candidate. We have already studied some factors and biomarkers of the coagulation in 150 COVID-19 patients, with interested results concerning thrombin generation or NETosis levels.

Finally, we have preliminary data from our cohort of patients with FXI deficiency suggesting a potential role of this disorder in transfusion related acute lung injury (TRALI). Preliminary results by our group found that two patients with congenital FXI deficiency who received transfusion of blood products developed TRALI. This finding has opened a new line of investigation as it suggests a new role for FXI.

Since variations in FXI levels may have clinical relevance, high FXI levels have been associated to thrombotic events (19) and FXI deficiency protects against thrombosis (13), some efforts have been done to identify the elements regulating FXI levels. These studies have evaluated the coding gene, *F11*, among patients with congenital deficiency or with increased levels (20). However, it is also possible other mechanisms might affect FXI levels. Other genes, including *KNG1* and miRNAs may modulate FXI levels (21–23). External factors may also influence FXI levels. In this framework we want to point out the identification of FXI inhibitors among cases with lupus anticoagulant (24–26). These data, the fact that FXI is a protein that binds to phospholipids, together with the requirements of new biomarkers that might help the clinical management of patients with antiphospholipid antibodies, a potentially severe disease due to its thrombotic complications (27), make this disorder an ideal target to study FXI.

Finally, the main limitation to study the contact system is the absence of sensitive and specific methods to evaluate the elements of the contact system. This is particularly relevant for the quantification of active forms, which *in vivo*, are expected to reach very low levels. The Contact System Network (COSYNE) is an initiative of the International Society of Thrombosis and Haemostasis (ISTH) leaded by Dr. C Maas, in which we participate, aiming to develop new methods using nanobodies to quantify free FXIIa, as well as PKa-C1INH; FXIa-C1INH, and FXIIa-C1INH complexes (https://academy.isth.org/isth/2020/isth-2020-virtual-congress-ssc-sessions/303254/coen.maas.cosyne.contact.system.network.html?f=menu=6*browseby=8*sortby=6*media=1*ce_id=1799*ces_id=26999*marker=793*featured=16856). Additionally,

there are some limitations concerning molecular aspects of *F11*. As far as we know, only SNVs or small in/dels have been described in cases with congenital FXI deficiency; no structural variants apart from a duplication described in our study have been described in these disorders (7). Moreover, the role that FXI-antisense gene (*F11-AS1*), contiguous to *F11* could have on the regulation of FXI levels or FXI deficiency, has never been studied. Finally, no regulatory studies including epigenetic control of these genes has been done to our knowledge.

Collaborators in the field: In Spain: 3 institutes (not described to not unravel the authorship). Among international experts in the contact system we point out three, all we are collaborating with: Dr David Gailiani (Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, Tennessee, USA.); Dr. Thomas Renné (Institute of Clinical Chemistry and Laboratory Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany), Dr. Jonas Emsley (Centre for Biomolecular Sciences, School of Pharmacy, University of Nottingham, Nottingham, NG7 2RD, England.) and Dr. Coen Maas (Department of Clinical Chemistry and Hematology, University Medical Center Utrecht, The Netherlands).

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Hypothesis

Despite there is a rebirth of the study on the contact system due to the new and fascinating information related to this system identified in the last 10 years, there are still many questions and challenges concerning FXI, a protein working as a crossroad between three key homeostatic systems: coagulation, inflammation and innate immunity. The identification and characterization of new variants affecting this protein may help to identify new key residues and domains in this molecule, new mechanisms involved in the transcriptional regulation, folding, secretion, and function, as well as to identify their potential clinical impact of this coagulopathy.

We also think that it is necessary to improve the methods (functional, antigenic and molecular) to study the elements of the contact system. These methods might be used to explore the consequences on the contact system of many disorders or situations that might potential this system, such as for example the use of artificial medical devices, or disorders with an important deregulation/activation of coagulation, inflammation and innate immunity such as COVID-19. Indeed, contact system elements may be new prognostic markers and even therapeutic targets in these disorders.

Objectives

The main objective of this project is to give new information on FXI, key elements of the contact system. The specific objectives of the project are:

1) To identify and characterize of new *F11* variants. Analysis of their clinical impact.
2) To develop new methods to study the contact system.
    a. New ELISAs based on nanobodies
    b. Molecular methods. Nanopore sequencing.
3) To characterize the contact system in different disorders.
    a. Cardiac valve replacement
    b. COVID-19
    c. TRALI
    d. Antiphospholipid antibodies
4) To study security outcomes for new antithrombotic drugs targeting the contact system by studying FXI deficient patients.

Methods

Subjects

The study will be done in a large cohort of patients with FXI variants recruited during the last 5 years (191 congenital FXI deficient patients). This project was approved the Ethics Committee of Hospital Reina Sofia. These patients were identified by molecular methods, directed by sequencing the coding genes or by massive sequencing methods (whole exome analysis). Table 1 shows the genetic variants identified so far in *F11* genes, respectively.

We remark one patient with aberrant FXI of unexpected high molecular weight (Figure 2).

Our study will continue including patients with FXI deficiency collected from different Spanish hospitals, identified by both clinical signs (bleeding) or by analysis of cases with prolonged aPTT after discarding other causes.

The contact system will be also evaluated in the following cohorts of patients:

a) A cohort of 155 COVID-19 patients who required hospitalization during the first wave in two Spanish hospitals (Morales Meseguer and Reina Sofia). Baseline and evolution samples were collected and stored at -80ºC. Clinical and laboratory data of these patients, including inflammation, and coagulation parameters has been collected. This project was approved by the Ethics Committee of Hospital Morales Meseguer.
b) A cohort of 320 consecutive patients underwent cardiac valve replacement using two procedures (Transcatheter aortic valve replacement –TAVR- and surgical aortic-valve replacement –SAVR-) in two Spanish hospitals (Hospital Clínico Universitario Virgen de la Arrixaca (Murcia), Hospital Alvaro Cunqueiro (Vigo)). The patients were recruited from 21/02/2018 to 11/03/2020 and from 15/02/2018 to 11/10/209, respectively Samples were collected 24 hours before and 48 hours after the replacement in vacuum tubes containing 0.109 M sodium citrate. Platelet-poor plasma (PPP) was

obtained within 2 hours after extraction by centrifugation (2000g x15 min) at 20°C and then stored at -80°C. Clinical and laboratory data of these patients, including inflammation, and coagulation parameters has been collected. This project was approved by the Ethics Committee of Hospital Clínico Universitario Virgen de la Arrixaca.

c) A cohort of patients with TRALI. A retrospective recruitment of patients who underwent fully documented TRALI in the Región de Murcia during the last 20 years will be done. Informed consent will be obtained for all patients enrolled in this study, which has to be approved by the Ethics Committee of Hospital Morales Meseguer.

d) A cohort of 194 consecutive patients with antiphospholipid antibodies recruited in a single hospital (Hospital Universitario Morales Meseguer) from January 2014 to June 2019. In this cohort, 112 patients had antiphospholipid syndrome and 82 patients were asymptomatic carriers. This project was approved by the Ethics Committee of Hospital Universitario Morales Meseguer.

Finally, the study will also include the analysis of liver samples from 15 subjects, 10 of them with non-alcoholic liver steatosis (with and without cirrhosis). Informed consent will be obtained for all patients enrolled in this study, which has to be approved by the Ethics Committee of Hospital Morales Meseguer.

As controls, we will study healthy controls (blood donors).

Identification and characterization of new FXI variants. Analysis of their clinical impact.

We will analyze plasma FXI from patients carrying genetic defects in the coding gene by using different methods:

a) Western blot using polyclonal antibody specific for human FXI (GAFXI-AP, goat, affinity purified IgG, Enzyme Research Laboratories).

b) Antigen levels by Luminex (Coagulation 6-Plex Human Panel 1, EPX060-10824-901, Antithrombin, CRP, Factor XI, Factor XII, Factor XIII, Prothrombin).
c) Coagulant activity (FXI:C), by using deficient plasmas (Factor XI deficient plasma HemosIL®) in automated coagulometers (ACL de Instrumentation Laboratory).
d) Activation of FXI by using silica and dextran sulfate. Chromogenic assays to determine the generation of FXIa.
e) Thrombin generation assays using calibrated automated thrombogram (CAT, Stago) activated by both tissue factor (1 and 5 pM) and silica.

For new cases recruited during this project, molecular analysis of *F11* gene will be done, originally by Sanger sequencing of coding exons, NGS, and MLPA when required.

Moreover, specific mutations will be expressed in a recombinant eukaryotic cell model (HEK 293 cells) after generating them by site directed mutagenesis in the plasmids containing the cDNA of human *F11*, kindly supplied by Drs. D Galiani. Analysis of wild type and recombinant FXI variants will be done in supernatant and in cell lysis samples.

Structural analysis of specific variants will be done in collaboration with Dr. J Emsley (Univeristy of Nottingham, UK) after their expression in insect cells.

1) Development or use of new methods to study the contact system.

a. New ELISAs based on nanobodies. We are participating in the COSYNE project of the ISTH who had provided the nanobodies to quantify by ELISA free FXIIa, as well as PKa-C1INH; FXIa-C1INH, and FXIIa-C1INH complexes. In addition to standardize the method, we would use these nanobodies to fully characterize baseline and activated samples of patients with *F11* genetic variants, and the disorders included in objective 3.
b. Molecular methods. Nanopore sequencing. Third generation sequencing methods allow not only to get long sequences but also characterize epigenetic

features of mRNA. We will use this technology, in collaboration with LongSeq applications S.L., to:

- Explore potential structural variants in these genes as cause of deficiency. This method will be complemented by MLPA.
- Evaluate the architecture of *F11-AS1* a gene encoding a F11 antisense close to *F11* whose function is unknown.
- Study the expression, alternative messengers, nucleotide sequence, and epigenetic changes of *F11, F11-AS1* mRNA from liver samples of patients with and without non-alcoholic liver steatosis.

2) Characterization of the contact system in different disorders.

FXI from plasma will be evaluated by the methods described in objectives 1 and 2, and molecular analysis of cases with aberrant or deficient FXI will be done in the cohorts previously described of the following disorders:

a. Cardiac valve replacement. Preliminary data suggest a reduction of FXI after the procedure, which would be related to the intervention and might have clinical impact as a prognostic biomarker.
b. COVID-19. Preliminary data obtained by an exome analysis in 87 young patients revealed two pathogenic heterozygous mutations in *F11*, one not previously described (c.692C>T:p.T231I) and secondly, much more interesting, probably causing an unusual CRM+ deficiency with potential dominant negative effect (FXI:C 37%) who was carried by a patient who suffered from a severe hemorrhage during COVID-19.
c. TRALI. Two patients with congenital FXI deficiency who were transfused developed severe TRALI. These preliminary data encourage studying a potential role for FXI (and its deficiency) in this immune disorder, which will be evaluated firstly by analysis of new cases with TRALI.

d. Antiphospholipid antibodies. Preliminary data support an increase of FXI among patients with antiphospholipid syndrome, and FXI deficiency due to both genetic and acquired causes in asymptomatic carriers. Indeed, we remark that we have identified patients with antiphospholipid antibodies carrying the Ashkenazy's more common mutation p.Glu135Stop. We aim to clarify whether the haplotype associated to this mutation is the same described in Ashkenazis.

3) Safety for new antithrombotic drugs targeting the contact system. The efficacy and safety of new anti-FXI drugs have mainly been tested in animal models. Any further information on potential side effects in humans can be achieved by analyzing particular situations in patients with FXI deficiency. In addition to the analysis of a potential exacerbation of the immune response that will be evaluated in objective 3-c (TRALI), we also aim to evaluate any interaction with other drugs interfering the hemostatic or inflammatory systems. Thus, we will explore potential side effects, bleeding, in patients with FXI deficiency treated with anticoagulant or antiplatelet, as well as with non-steroids anti-inflammatory drugs.

Strengths of the research team

The team has been working in the contact system during the last 7 years, recruiting a large cohort of patients with genetic variants affecting FXI, some of them rendering aberrant molecules.

The team is multidisciplinary including basic and clinical investigators, and has collaborations of excellence with national and international expert in the field.

Table 1. Genetic variants identified in *F11* gene in unrelated families.

Genetic variant	N unrelated families	Type of deficiency	Reference	Comments
p.C56R	Homozygosis N= 3 Heterozygosis N= 28 Compound heterozygosis N= 1	CRM-	CM020681	
p.A91T	Heterozygosis N=1	CRM-	CS081910	
p.E131*	Heterozygosis N= 2 Compound heterozygosis N= 2	CRM-	CM890042	Common in Ashkenazys
p.T231I	Heterozygosis N=1	CRM-	**NEW**	
p.Q244R	Heterozygosis N=1	**CRM+**	CM980651	
p.C255Y	Heterozygosis N=1	CRM-	CM020682	
p.R268C	Heterozygosis N=2	CRM-	CM035499	
p.R268H	Heterozygosis N=1	**CRM+**	CM083501	
p.F295C	Compound heterozygosis N=1	CRM-	CM020682	Aberrant FXI
p.T322I	Heterozygosis N=2	CRM-	CM950373	
p.C339F	Compound heterozygosis N=1	CRM-	**NEW**	
p.C416Y	Heterozygosis N=9 Compound heterozygosis N=2	CRM-	CM053240	
p.I426T	Heterozygosis N=2	CRM-	**NEW**	
p.R443C	Heterozygosis N=2	CRM-	CM062624	
p.K536N	Compound heterozygosis N=1	**CRM+**	CM002953	
p.P538L	Homozygosis N= 1 Heterozygosis N=1	**CRM+**	CM051916	
p.E565K	Heterozygosis N=6	CRM-	CM051917	
p.I592T	Heterozygosis N=1	CRM-	**NEW**	
p.C599Y	Compound heterozygosis N=1	CRM-	**NEW**	
Duplication of exons 8-9	Heterozygosis N=1	CRM-	**NEW**	
Complete deletion of F11	Compound heterozygosis N=1	CRM-	**NEW**	*De novo* mutation

Figure 1. Scheme of the crosstalk roles of FXI in three systems: coagulation cascade, innate immunity and inflammation.

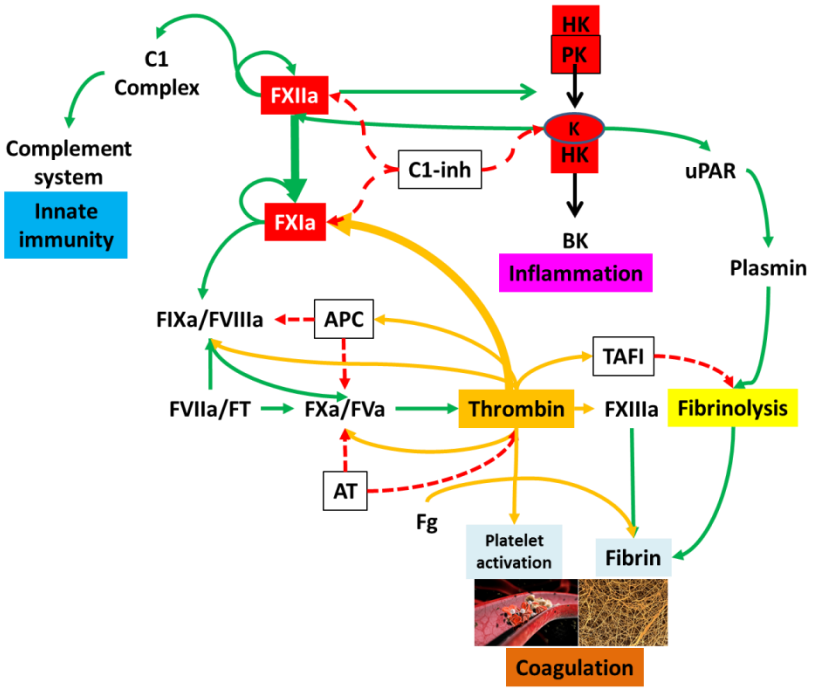

Figure 2. Patient with aberrant FXI.