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Molecular Characterisation of 11 novel mutations in patients with heterozygous and homozygous FV deficiency.

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Running title: 11 novel mutations in FV deficient patients

Key words: F5, Mutation, FV deficiency

Abstract:

Introduction: Coagulation factor V (FV) has an important role in the blood coagulation cascade, in both the pro- and anticoagulant pathways. FV deficiency is a rare bleeding disorder with variable phenotypic expression.

Aim: We report a cohort of 10 patients with mild- severe FV deficiency in whom a total of 11 novel mutations were identified.

Results: Three patients were compound heterozygous for two mutations while each of the remaining patients had a single heterozygous variant. FV levels did not correlate with either the type of mutation identified or the bleeding diathesis exhibited by the patients.

Conclusion: Although considered to have an autosomal recessive mode of inheritance, patients with a single missense mutation may present with a significant bleeding history. The addition of a significant number of previously unidentified mutations to the public domain will contribute to the knowledge and understanding of the molecular pathology of this rare disorder.

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Introduction:

Blood coagulation factor V (FV) is a protein cofactor, an essential element in the rapid generation of thrombin catalysed by the prothrombinase cascade [1]. It also participates in the down regulation of factor VIII activity by enhancing the effect of Activated Protein C [2], thus having a role in both the procoagulant and anticoagulant pathways. The clinical effect of the activated FV cofactor depends on the local environment: the relative concentrations of pro- and anti-coagulant enzymes. FV deficiency is a rare disorder, with an incidence of approximately 1:1 million [3]. It was first reported by Owrens in 1943 [4] and has been extensively studied in the intervening decades [5]. Clinical symptoms range from mild to severe, and include epistaxis, menorrhagia, and post traumatic bleeding. Haematomas and haemarthroses occur in approximately 25% of cases, and life threatening bleeds in the central nervous system or gastrointenstinal tract are rare [6]. Type I FV deficiency (a quantitative defect with low or absent antigen) is much more commonly reported (over 200 cases in the literature [7] than Type II deficiency (a qualitative deficiency showing normal / mildly reduced antigen levels and very low activity) for which there are only 2 reported associated mutations (Ala221Val, Arg2080Cys)[8,9]. A thrombotic tendency is demonstrated in a minority of cases [10], and in a number of cases results from a pseudo-homozygous FVL state- the combination of a null allele and the in trans expression of the thrombotic risk factor FV Leiden (Arg506Gln) [11]. Although classically defined as having an autosomal recessive mode of inheritance, patients with a single heterozygous mutation generally present with mild to moderate type I FV deficiency. Typically these patients have levels >20%, and are usually asymptomatic [12]. Patients with the HR2 haplotype (a group of linked polymorphic FV loci) may present with a mild reduction in FV levels [13].

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The gene coding for human FV (*F5*) maps to chromosome 1q24.2. It spans 74.5kb [14], and comprises 25 exons [15]. The domain structure of FV (A1-A2-B-A3-C1-C2) is similar to that of FVIII [16], and indeed there is 35-40% homology between the A and C domains of FV and FVIII. The three A domains also have significant homology with the triplicated A domains of Ceruloplasmin [17]. The B domain of FV, which shares no sequence similarity with any known protein, is removed by proteolytic cleavage by thrombin, resulting in activation of the FV procofactor to FVa [18]. A similar cleavage event removes the B domain of FVIII [19], further demonstrating the similarities between these two cofactors.

The first mutation in the *F5* gene was reported in 1998[20] , and since then there have been a number of additional reports of *F5* variants and their association with FV deficiency in both symptomatic patients and those who show no increased tendency to bleed [21-23].

In this study we report 11 novel mutations in 11 unrelated patients from two centres with phenotypic and clinical characteristics of FV deficiency.

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Patients and Methods:

Patients:

The study group comprises of 10 patients with an age range 18-48 years. All patients presented with prolonged Prothrombin time (PT), Activated Partial Thromboplastin Time (APTT) and reduced FV: C. All patients suffered from a moderate to severe

bleeding and easy bruising tendency. Additional family members were available for study in a small subset of this patient group.

Methods:

Factor V activity and FV antigen:

FV clotting activity (FV:C) was assayed by a one stage coagulation assay based on the prothrombin time using parallel line bioassay analysis, performed on a Sysmex CA1500 analyser (Sysmex UK, Milton Keynes, U.K.) FV antigen levels (FV: Ag) were estimated by sandwich ELISA performed on a Rosys PLATO analyser.

Genotypic Analysis:

Following informed consent, EDTA anticoagulated blood samples were collected. DNA was isolated using a commercial DNA extraction kit (Qiagen, Crawley, UK). Additionally, for some patients blood was drawn into a commercial RNA stabilising solution (PAXgene, Qiagen)

All 25 exons of the *F5* gene were PCR amplified by standard techniques (PCR primer sequences are available on request). Bi-directional sequencing was performed using BigDye terminator chemistry (v3.1) on an ABI Prism® 3130xl genetic analyser (Applied Biosystems, Warrington, U.K.) All primers were designed based on the

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sequence of *F5* NG_011806.1 and were checked for single nucleotide polymorphisms using SNPCheck software (http://ngrl.man.ac.uk/SNPCheck)

Reverse transcription-PCR:

Reverse transcription-PCR (RT-PCR) was performed using commercially available Reverse Transcription Reagents Kit from Applied Biosystems. Following reverse transcription, the cDNA was subjected to PCR amplification, using primers designed to *F5* NM_000130.4 and the product analysed by sequencing with BigDye v3.1 terminator chemistry.

Results:

All patients showed a reduction in FV activity levels (FV:C) ranging from <1 to 48.3 (normal range 66-114 iu/dl). FV antigen levels were available in a minority of the patients, and where measured showed an approximately equivalent reduction to their activity levels, ranging from 5 to 22 (normal range FV:Ag 53-110 iu/dl). These levels were consistent with the diagnosis of FV deficiency. FVIII: C levels were reported in all patients, and lay within normal range, thereby excluding the differential diagnosis of combined FV+VIII deficiency. In all cases where FV: C levels were above 25iu/dl patients were genetically heterozygous, while those patients with severely reduced

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levels (<10iu/dl) were shown to be compound heterozygous for two mutations. Where additional family members were available, the identified mutations were demonstrated to co-segregate with the disease.

Mutation screening was performed on all 10 patients, by sequencing the 25 exons of the *F5* gene, including the exon-intron boundaries. Eleven different heterozygous sequence variants were identified. These were cross referenced with the on line database of mutations and polymorphisms in the FV gene (http://www.lumc.nl/rep/cod/redirect/4010/research/factor_v_gene.html), and none were found to have been previously described. Details of the mutations identified are given in table 1. Three nonsense mutations were detected which, due to their location within the gene, are postulated to result in nonsense mediated decay rather than the production of a truncated protein. Two putative splicing variants were identified: One, heterozygosity for the splicing of exon 7 to c.1118 +1278 was detected only after reverse transcription of RNA. This aberrant splicing results in an increase in the number of amino acid residues in the A2 domain, which is the interactive region between FV and FIIa. The genomic cause of this aberrant splicing could not be determined. The second splicing abnormality, c.250+1G>A is predicted to abolish the IVS2 donor splice site. Although this has not been confirmed at the RNA level, this nucleotide is part of the invariant GT dinucleotide [24] and its effect can be predicted with confidence. In one patient, the duplication of 78 nucleotides predicts the inframe insertion of an additional 26 amino acids into the A1 domain. Five missense mutations were identified: Leu90Ser, Gly259Glu, Arg1659Pro, His 1803Arg and Leu1849Ser. All are predicted to be pathogenic by *in silico* analysis.

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Three patients were found to be compound heterozygous for two mutations, and two of the variants were found in more than one unrelated proband. In addition to the mutations described in this report, all patients also demonstrated heterozygosity for polymorphic changes known to be present in the general population.

Discussion:

Factor V deficiency is a rare inherited bleeding disorder which, even in patients with a severe phenotype of very low or unmeasurable plasma FV levels, frequently manifests with mild clinical symptoms. We have studied 10 patients with mild to severe factor V deficiency, showing little correlation with their clinical symptoms: ranging from none (asymptomatic male with FV:C 6 iu/dl) to post operative and post partum bleeding (female patient with FV:C 43 iu/dl). None of these patients had any of the confirmed or putative pro-thrombotic risk factors within the *F5* gene (FV Leiden [25], FV Cambridge [26] FV Hong Kong [27], or components of the HR2 haplotype) which could affect the clinical picture.

Sequencing of the *F5* gene in this group of patients identified 11 previously unreported mutations (2 of which were seen in more than one patient). Three nonsense mutations, one in-frame insertion, two splicing defects and five missense mutations were located in the A1, A3 and C2 domains- with one premature termination codon being situated in the B domain. The in- frame duplication of 26 amino acids was identified in the A1 domain of one patient, who also has congenital

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mild FX deficiency (FX:C 34.6 iu/dl, associated with heterozygosity for a p.Gly134Arg substitution) The distribution of missense mutations reflects that of previously described *F5* mutations. The paucity of missense mutations from the B domain of *F5* and *F8* is unsurprising, and probably relates to its tolerance to amino acid variation, demonstrated by its polymorphic nature and its lack of conservation. The need for a highly regulated B domain is minimised as it is excised during FV activation. Novel missense variants in the B domain should be interpreted with caution before the assignment of pathogenicity. The IVS2+1G>A splicing variant was assessed using two *in silico* splice predictors, each of which predicted the abolition of the donor splice site. It has not been determined whether the two recurrent mutations (His1803Arg and Leu1849Ser) arose independently or are of ancestral origin.

In silico analysis of the five missense mutations was undertaken, utilising on –line available SIFT (http://blocks.fhcrc.org/sift/SIFT.html), PolyPhen (http://genetics.bwh.harvard.edu/pph/), PMut (http://mmb2.pcb.ub.es:8080/PMut/) and Russell Analysis (http://www.russell.embl-heidelberg.de/aas/) programs. Additionally the absence of all variants was confirmed on both ENSEMBL (http://www.ensembl.org/) and Seattle SNPs (http://pga.gs.washington.edu/) websites. The analysis tools predict the possible impact of an amino acid substitution on protein structure and function, based on comparisons between amino acid properties, protein homologies and evolutionary conservation. The four analysis tools gave concordant conclusions for only 2/5 missense mutations (Gly259Glu and Leu1849Ser). For each of the other three variants one tool gave a conflicting prediction, although in each case the pattern of agreed results differed. These discrepancies demonstrate the limitations of *in silico* analysis, and highlight the need for thorough examination before associating a novel variant with FV deficiency. Protein modelling was undertaken,

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using SwissProtDeepView software to effect the amino acid substitution and Protein Explorer to visualise any local structural alterations. The native glycine at position 259 is a small residue located at the interaction between the A1 and A3 domains (fig 1C). Its replacement with the large negatively charged glutamine residue (fig 1D) may alter native domain interactions by both spatial and charge differences, as the glutamine will prefer a buried position [28]. The function of the very large arginine residue at position1631 (fig 1E) may be to maintain local stability. This is likely to be negatively affected by its substitution with the small neutral proline (fig1F) introducing a tight turn which produces gross conformational changes. Histidine 1803 (fig 1G) is a polar residue in the A3 domain. The substitution of this residue with arginine confers a change in charge to the region, and alters local interactions (fig 1H). The effect of this mutation is severe, as seen in patient 9, with almost immeasurable FV levels, where it is seen in compound heterozygosity with a premature stop codon, encoding a null allele. The hydrophobic leucine residue at position 1849 (fig 1I) is located in the A3 domain. Its replacement with a hydrophilic serine residue (fig 1J) will result in conformational changes which may affect interaction with the A1 domain. In contrast, the same amino acid substitution in the A1 domain (Leu90Ser) is predicted to result in a limited conformational change (figs1A and 1B) possibly due to the relatively neutral properties of serine. The pathogenicity of the Leu90Ser substitution must remain putative at present, but further investigations may elicit additional insights into its function. The limited structural damage may be suggestive of a type II mutation, with discrepant FV antigen and activity levels, although as yet FV Ag levels have not been assessed on this patient. Expression of the serine90 variant and generation of a mutant protein would offer further support to its pathogenic function. Although the Leu90Ser mutation is the only

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variant identified in this patient (other than known benign polymorphisms) its co-segregation with mild FV deficiency in this family cannot be proven as reports of a bleeding tendency in previous generations in the paternal line are anecdotal. Whilst the index case has a life long history of easy bruising and trauma associated bleeding all her living first degree relatives are asymptomatic. Whilst extensive laboratory investigations coupled with *in silico* analysis is generally sufficient to label a previously unidentified variant as a mutation, caution should always be exercised in assigning pathogenicity: the definitive tool is considered to be i*n vitro* expression of the mutant protein in cell culture. In some cases a variant may be better described as a marker than as causative of the deficiency.

The number and type of mutations reported here are broadly consistent with the spectrum seen in other studies. Point mutations and frameshift mutations predominate, and a small number of splice variants have been detected.

The number and spectrum of mutations identified within the F5 gene is increasing rapidly as more families are studied. Each report of a novel mutation adds to our understanding of this gene, and the function of this complex protein with its dual pro- and anti-coagulant functions.

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Patient id	Nucleotide variant[a]	Predicted effect on protein[b]	Domain	FV:C[c]	FV:Ag[d]
1	Het c.269T>C	p.Leu90Ser (Leu62Ser)	A1	43	
2	Het c.683-760dup78	p.201-226dup (173-198dup)	A1	41.4	
3	Het c.706G>T	p.Gly236X (Gly208X)	A1	37	
4	Het c.776G>A	p.Gly259Glu (Gly231Glu)	A1	6	5.6
	Het splice exon 7 to IVS7+1278	Aberrant splicing	-		
5	Het c.250 +1 G>A	Loss of intron 2 donor splice site	-	29.7	
6	Het c.2772G>A	p.Trp924X (Trp896X)	B	36	22
7	Het c.4975G>C	p.Arg1659Pro (Arg1631Pro)	A3	41.3	
8	Het c. 5408A>G	p.His1803Arg (His 1775Arg)	A3	2.5	5
	Het c.5546T>C	p.Leu1849Ser (Leu1821Ser)	A3		
9	Het c.5408A>G	p.His1803Arg (His1775Arg)	A3	<1	
	Het c.6257C>A	p.Ser2086X (Ser2058X)	C2		
10	Het c.5546T>C	p.Leu1849Ser (Leu1821Ser)	A3	35	

Table 1 Genotypic and phenotypic data of FV deficient patients

[a] Nucleotide numbering according to HGVS nomenclature where 1 is the A of the initiating Methionine. The reference sequence is NM_000130.4

[b] Amino acid numbering according to HGVS nomenclature where 1 is the initiating Methionine. The reference sequence is NP_000121.2. The nomenclature in parenthesis is according to Jenny *et al.* [13]

[c] Normal Range 66-114 iu/dl

[d] Normal Range 53-110 iu/dl

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Fig 1: Comparison of structural differences between wild type and mutant variants Models were created using Protein Explorer software, with variant forms being produc SwissProt Deep-View software. The models are presented as ribbon diagrams with the relevant amino acid in each case being shown in 'space fill' format. The domains in each display are oriented to best display the site of the mutated residue. Fig 1A+B represents Leu90Ser, 1C+D are the Gly259Glu models, with the A1 and A3 domains labelled. 1E +F shows Arg1659 Pro, 1G+H are the His1803Arg variants and 1I+J theLeu1849Ser. In each pairing the wild type model is presented first.

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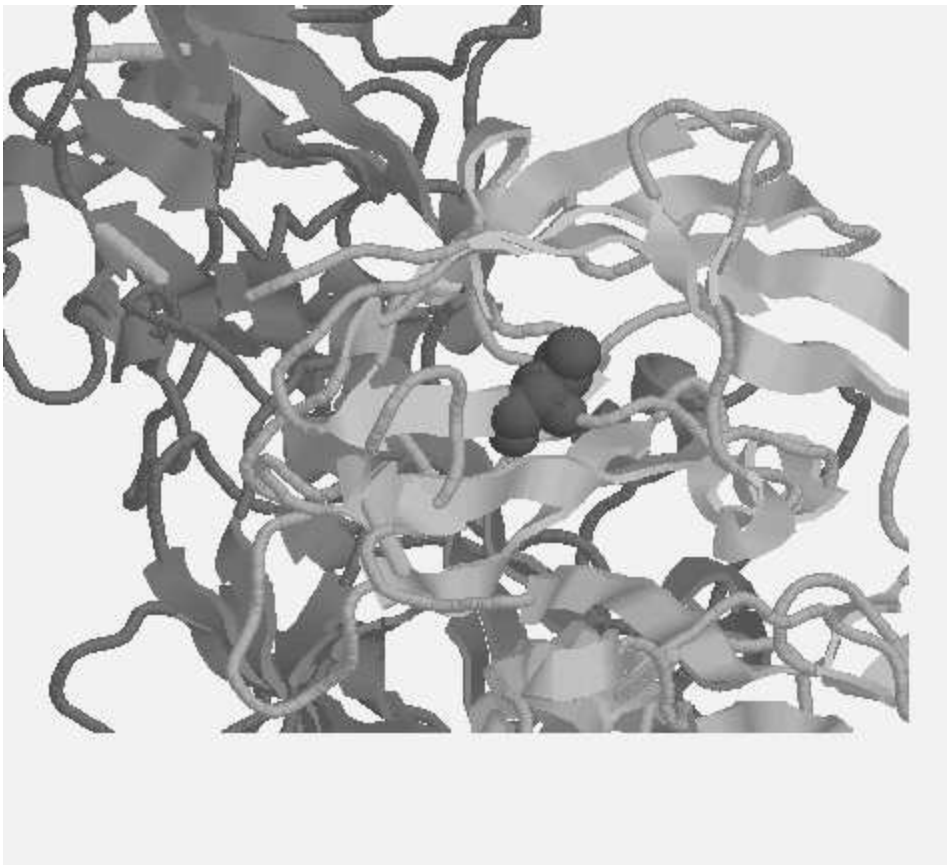

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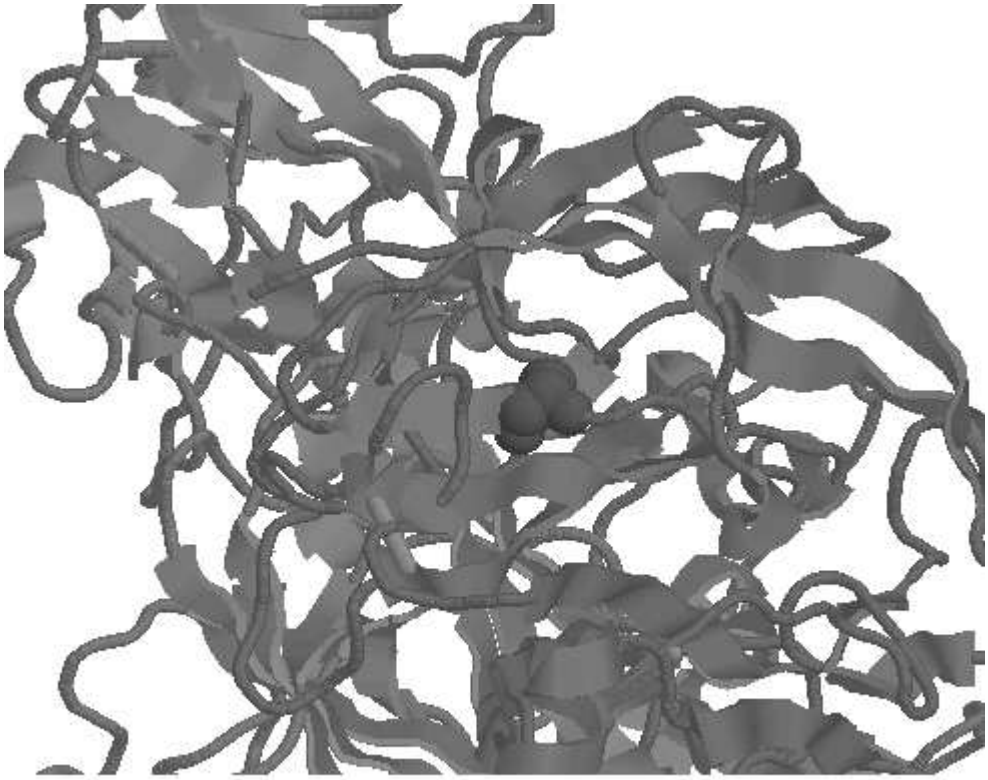
View



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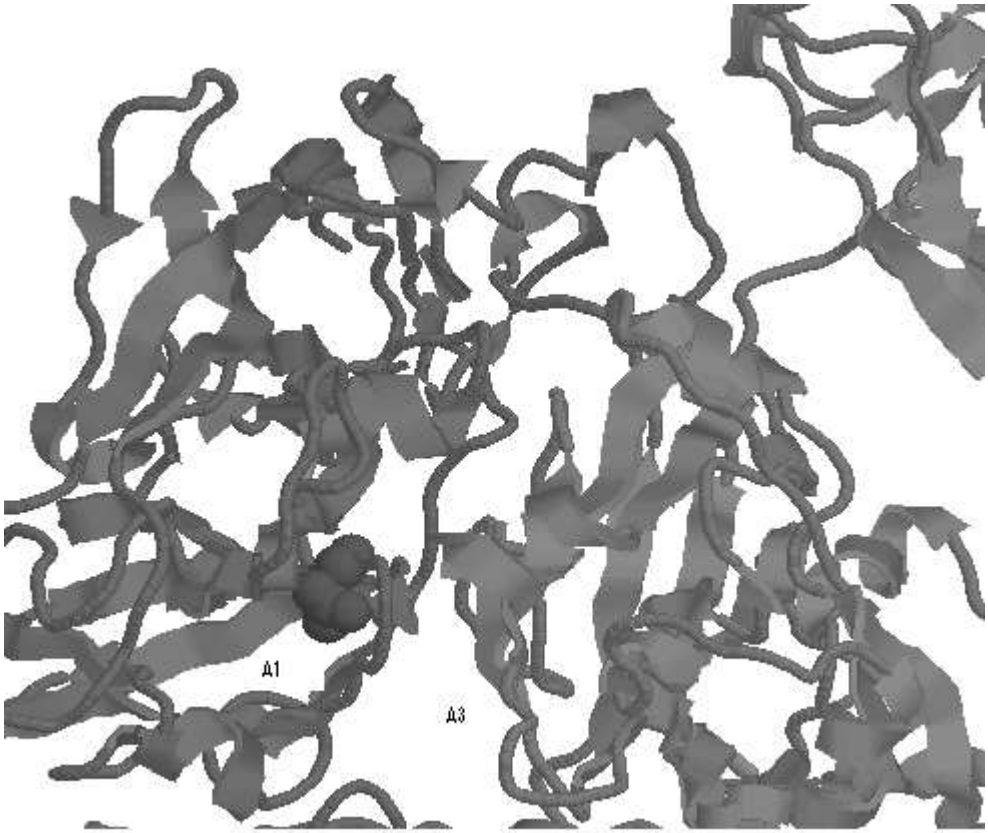

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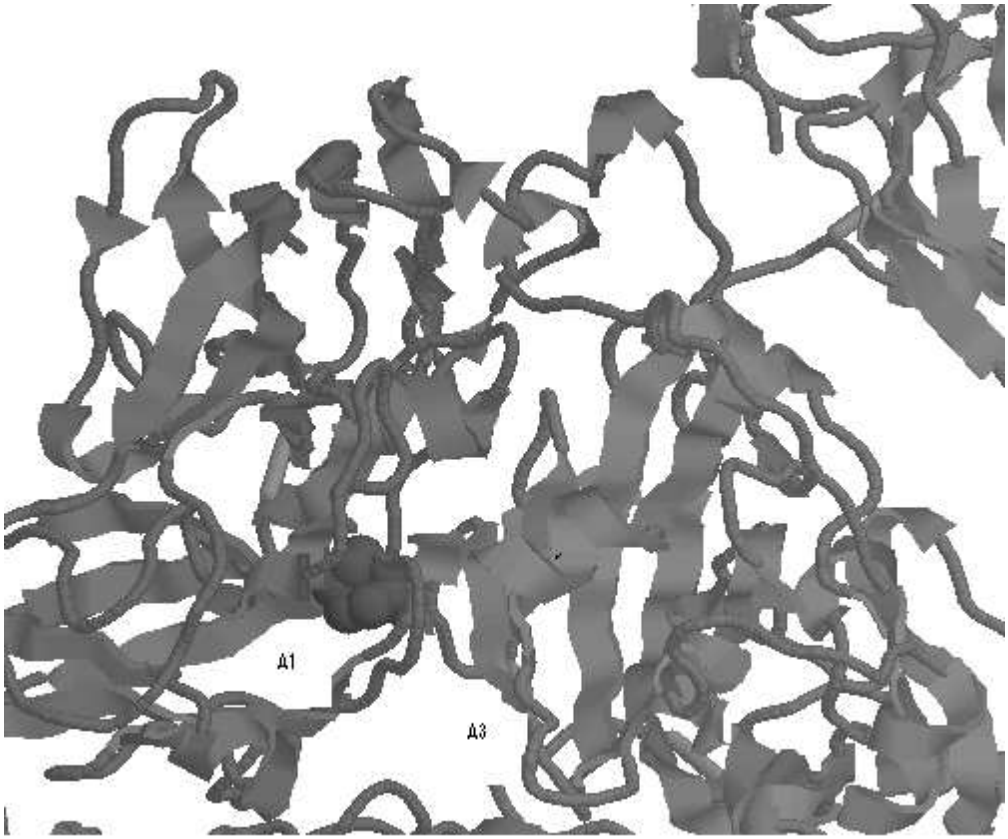

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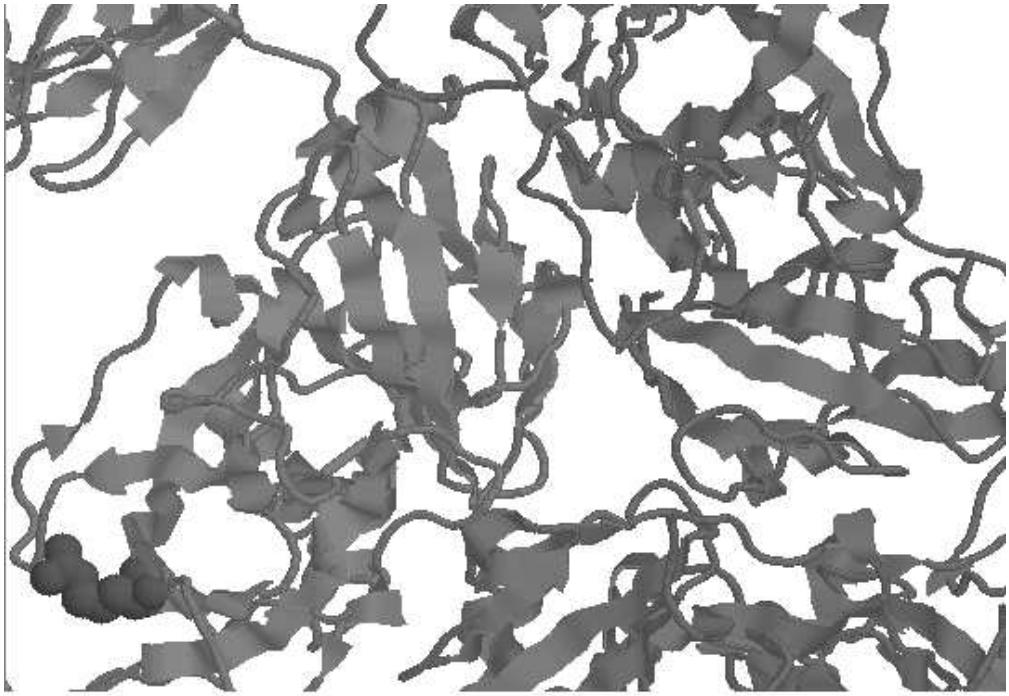

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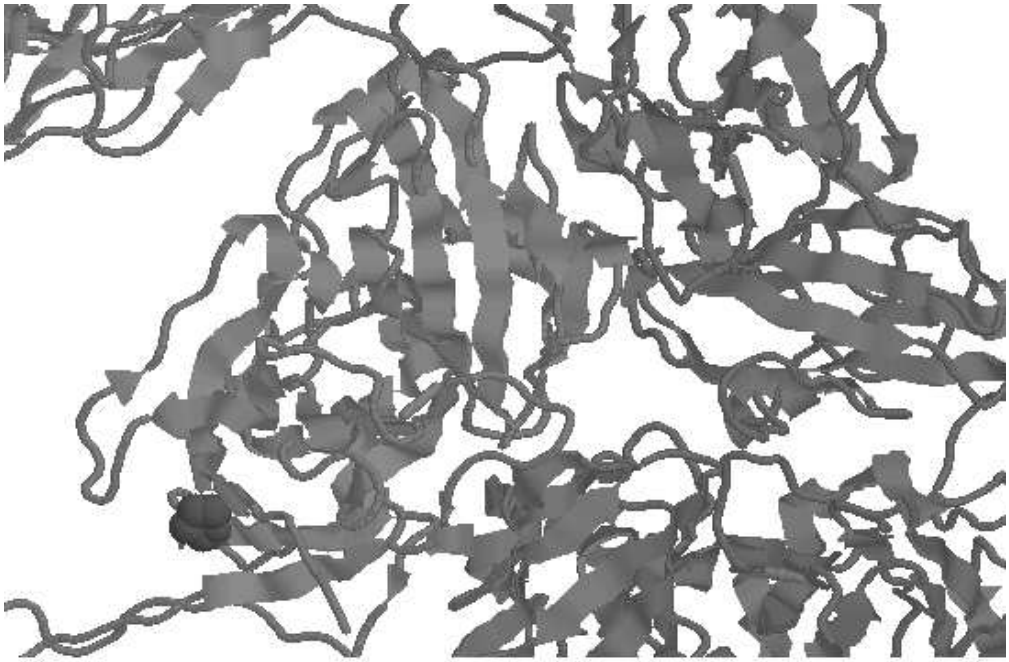

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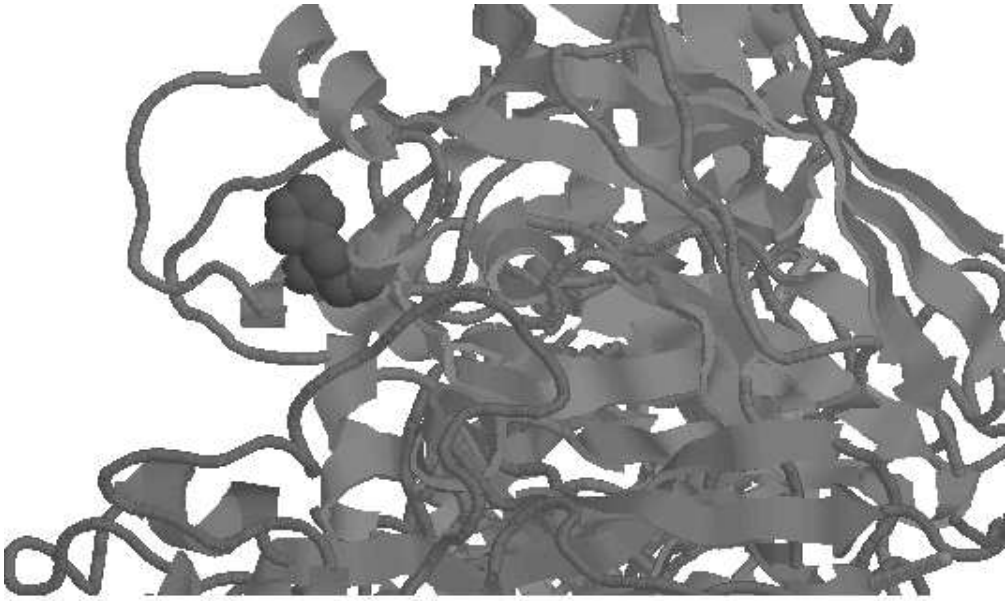

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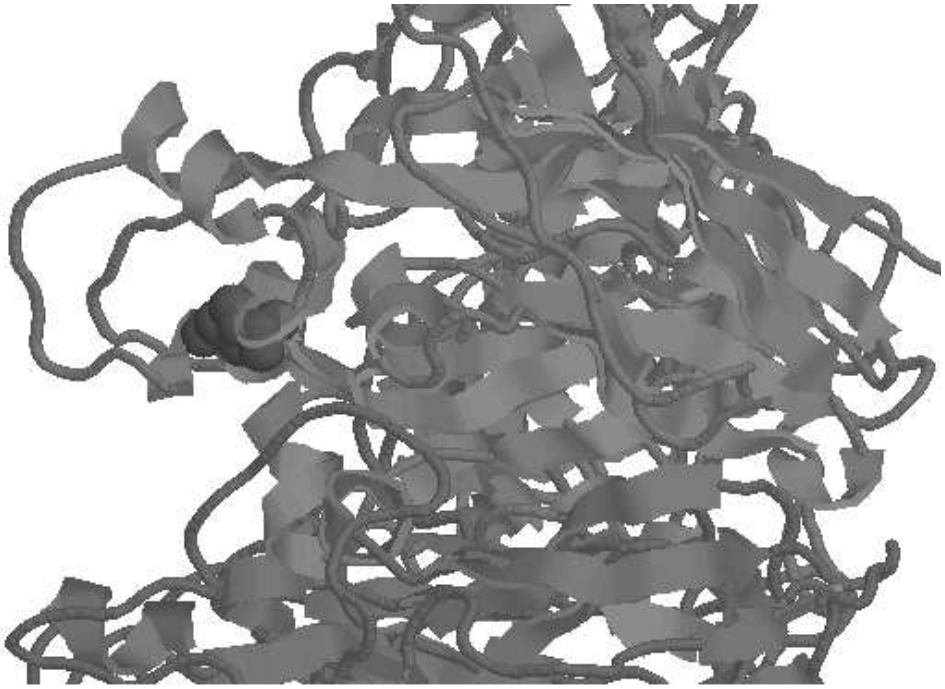

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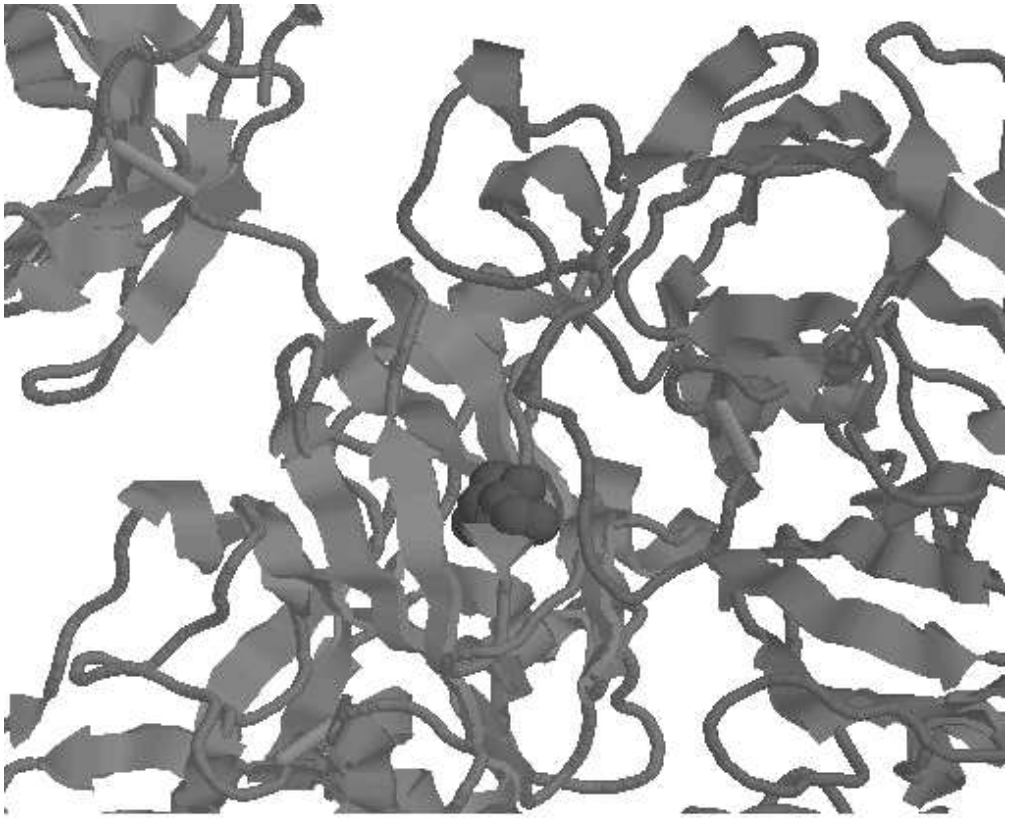

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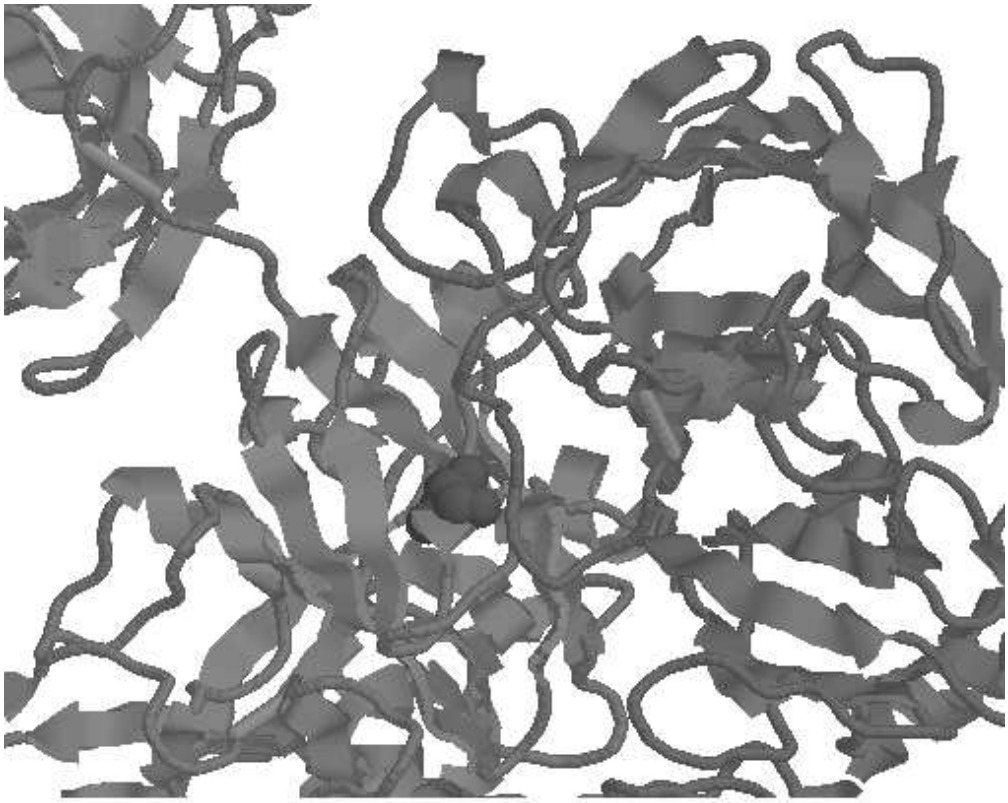

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08 March 2010

Re: Molecular Characterisation of 11 novel mutations in patients with heterozygous and homozygous FV deficiency.

Please accept this manuscript for consideration of publication in the journal Haemophilia. The report is on a number of novel mutations identified in a group of FV deficient patients from 2 centres in the UK. The work is original, and is not under consideration of publication by any other journal. All 5 authors have read and approved the manuscript and none have any conflict of interest to report.

Yours faithfully

On behalf of the authors

Jacqueline Cutler

Centre for haemostasis and Thrombosis

For Peer Review