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

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

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3 **Molecular Characterisation of 11 novel mutations in patients with**
4 **heterozygous and homozygous FV deficiency.**
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53 Running title: 11 novel mutations in FV deficient patients
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55 Key words: F5, Mutation, FV deficiency
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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.

Introduction:

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3 Blood coagulation factor V (FV) is a protein cofactor, an essential element in the
4 rapid generation of thrombin catalysed by the prothrombinase cascade [1]. It also
5
6 participates in the down regulation of factor VIII activity by enhancing the effect of
7
8 Activated Protein C [2], thus having a role in both the procoagulant and anticoagulant
9
10 pathways. The clinical effect of the activated FV cofactor depends on the local
11
12 environment: the relative concentrations of pro- and anti-coagulant enzymes. FV
13
14 deficiency is a rare disorder, with an incidence of approximately 1:1 million [3]. It
15
16 was first reported by Owrens in 1943 [4] and has been extensively studied in the
17
18 intervening decades [5]. Clinical symptoms range from mild to severe, and include
19
20 epistaxis, menorrhagia, and post traumatic bleeding. Haematomas and haemarthroses
21
22 occur in approximately 25% of cases, and life threatening bleeds in the central
23
24 nervous system or gastrointestinal tract are rare [6]. Type I FV deficiency (a
25
26 quantitative defect with low or absent antigen) is much more commonly reported
27
28 (over 200 cases in the literature [7]) than Type II deficiency (a qualitative deficiency
29
30 showing normal / mildly reduced antigen levels and very low activity) for which there
31
32 are only 2 reported associated mutations (Ala221Val, Arg2080Cys)[8,9]. A
33
34 thrombotic tendency is demonstrated in a minority of cases [10], and in a number of
35
36 cases results from a pseudo-homozygous FVL state- the combination of a null allele
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38 and the in trans expression of the thrombotic risk factor FV Leiden (Arg506Gln) [11].
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40 Although classically defined as having an autosomal recessive mode of inheritance,
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42 patients with a single heterozygous mutation generally present with mild to moderate
43
44 type I FV deficiency. Typically these patients have levels >20%, and are usually
45
46 asymptomatic [12]. Patients with the HR2 haplotype (a group of linked polymorphic
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48 FV loci) may present with a mild reduction in FV levels [13].
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3 The gene coding for human FV (*F5*) maps to chromosome 1q24.2. It spans 74.5kb
4 [14], and comprises 25 exons [15]. The domain structure of FV (A1-A2-B-A3-C1-C2)
5 [14], and comprises 25 exons [15]. The domain structure of FV (A1-A2-B-A3-C1-C2)
6 [14], and comprises 25 exons [15]. The domain structure of FV (A1-A2-B-A3-C1-C2)
7 is similar to that of FVIII [16], and indeed there is 35-40% homology between the A
8 and C domains of FV and FVIII. The three A domains also have significant homology
9 with the triplicated A domains of Ceruloplasmin [17]. The B domain of FV, which
10 shares no sequence similarity with any known protein, is removed by proteolytic
11 cleavage by thrombin, resulting in activation of the FV procofactor to FVa [18]. A
12 similar cleavage event removes the B domain of FVIII [19], further demonstrating the
13 similarities between these two cofactors.
14

15 The first mutation in the *F5* gene was reported in 1998[20] , and since then there have
16 been a number of additional reports of *F5* variants and their association with FV
17 deficiency in both symptomatic patients and those who show no increased tendency to
18 bleed [21-23].
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20 In this study we report 11 novel mutations in 11 unrelated patients from two centres
21 with phenotypic and clinical characteristics of FV deficiency.
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34 **Patients and Methods:**

35 **Patients:**

36 The study group comprises of 10 patients with an age range 18-48 years. All patients
37 presented with prolonged Prothrombin time (PT), Activated Partial Thromboplastin
38 Time (APTT) and reduced FV: C. All patients suffered from a moderate to severe
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3 bleeding and easy bruising tendency. Additional family members were available for
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5 study in a small subset of this patient group.
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10 11 12 13 14 15 **Methods:**

16 17 18 19 20 **Factor V activity and FV antigen:**

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22 FV clotting activity (FV:C) was assayed by a one stage coagulation assay based on
23
24 the prothrombin time using parallel line bioassay analysis, performed on a Sysmex
25
26 CA1500 analyser (Sysmex UK, Milton Keynes, U.K.) FV antigen levels (FV: Ag)
27
28 were estimated by sandwich ELISA performed on a Rosys PLATO analyser.
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41 42 **Genotypic Analysis:**

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44 Following informed consent, EDTA anticoagulated blood samples were collected.
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46 DNA was isolated using a commercial DNA extraction kit (Qiagen, Crawley, UK).
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48 Additionally, for some patients blood was drawn into a commercial RNA stabilising
49
50 solution (PAXgene, Qiagen)
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53 All 25 exons of the *F5* gene were PCR amplified by standard techniques (PCR primer
54
55 sequences are available on request). Bi-directional sequencing was performed using
56
57 BigDye terminator chemistry (v3.1) on an ABI Prism® 3130xl genetic analyser
58
59 (Applied Biosystems, Warrington, U.K.) All primers were designed based on the
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3 sequence of *F5* NG_011806.1 and were checked for single nucleotide polymorphisms
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5 using SNPCheck software (<http://ngrl.man.ac.uk/SNPCheck>)
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10 11 12 13 14 15 16 17 18 **Reverse transcription-PCR:**

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22 Reverse transcription-PCR (RT-PCR) was performed using commercially available
23
24 Reverse Transcription Reagents Kit from Applied Biosystems. Following reverse
25
26 transcription, the cDNA was subjected to PCR amplification, using primers designed
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28 to *F5* NM_000130.4 and the product analysed by sequencing with BigDye v3.1
29
30 terminator chemistry.
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40 **Results:**

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45 All patients showed a reduction in FV activity levels (FV:C) ranging from <1 to 48.3
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47 (normal range 66-114 iu/dl). FV antigen levels were available in a minority of the
48
49 patients, and where measured showed an approximately equivalent reduction to their
50
51 activity levels, ranging from 5 to 22 (normal range FV:Ag 53-110 iu/dl). These levels
52
53 were consistent with the diagnosis of FV deficiency. FVIII: C levels were reported in
54
55 all patients, and lay within normal range, thereby excluding the differential diagnosis
56
57 of combined FV+VIII deficiency. In all cases where FV: C levels were above 25iu/dl
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59 patients were genetically heterozygous, while those patients with severely reduced
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3 levels (<10iu/dl) were shown to be compound heterozygous for two mutations.

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5 Where additional family members were available, the identified mutations were
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7 demonstrated to co-segregate with the disease.
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12 Mutation screening was performed on all 10 patients, by sequencing the 25 exons of
13
14 the *F5* gene, including the exon-intron boundaries. Eleven different heterozygous
15
16 sequence variants were identified. These were cross referenced with the on line
17
18 database of mutations and polymorphisms in the FV gene
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20 (http://www.lumc.nl/rep/cod/redirect/4010/research/factor_v_gene.html), and none
21
22 were found to have been previously described. Details of the mutations identified are
23
24 given in table 1. Three nonsense mutations were detected which, due to their location
25
26 within the gene, are postulated to result in nonsense mediated decay rather than the
27
28 production of a truncated protein. Two putative splicing variants were identified: One,
29
30 heterozygosity for the splicing of exon 7 to c.1118 +1278 was detected only after
31
32 reverse transcription of RNA. This aberrant splicing results in an increase in the
33
34 number of amino acid residues in the A2 domain, which is the interactive region
35
36 between FV and FIIIa. The genomic cause of this aberrant splicing could not be
37
38 determined. The second splicing abnormality, c.250+1G>A is predicted to abolish the
39
40 IVS2 donor splice site. Although this has not been confirmed at the RNA level, this
41
42 nucleotide is part of the invariant GT dinucleotide [24] and its effect can be predicted
43
44 with confidence. In one patient, the duplication of 78 nucleotides predicts the inframe
45
46 insertion of an additional 26 amino acids into the A1 domain. Five missense
47
48 mutations were identified: Leu90Ser, Gly259Glu, Arg1659Pro, His 1803Arg and
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60 Leu1849Ser. All are predicted to be pathogenic by *in silico* analysis.

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

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27 Factor V deficiency is a rare inherited bleeding disorder which, even in patients with a
28 severe phenotype of very low or unmeasurable plasma FV levels, frequently manifests
29 with mild clinical symptoms. We have studied 10 patients with mild to severe factor
30 V deficiency, showing little correlation with their clinical symptoms: ranging from
31 none (asymptomatic male with FV:C 6 iu/dl) to post operative and post partum
32 bleeding (female patient with FV:C 43 iu/dl). None of these patients had any of the
33 confirmed or putative pro-thrombotic risk factors within the *F5* gene (FV Leiden [25],
34 FV Cambridge [26] FV Hong Kong [27], or components of the HR2 haplotype) which
35 could affect the clinical picture.
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48 Sequencing of the *F5* gene in this group of patients identified 11 previously
49 unreported mutations (2 of which were seen in more than one patient). Three
50 nonsense mutations, one in-frame insertion, two splicing defects and five missense
51 mutations were located in the A1, A3 and C2 domains- with one premature
52 termination codon being situated in the B domain. The in- frame duplication of 26
53 amino acids was identified in the A1 domain of one patient, who also has congenital
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3 mild FX deficiency (FX:C 34.6 iu/dl, associated with heterozygosity for a
4 p.Gly134Arg substitution) The distribution of missense mutations reflects that of
5
6 previously described *F5* mutations. The paucity of missense mutations from the B
7
8 domain of *F5* and *F8* is unsurprising, and probably relates to its tolerance to amino
9
10 acid variation, demonstrated by its polymorphic nature and its lack of conservation.
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12 The need for a highly regulated B domain is minimised as it is excised during FV
13
14 activation. Novel missense variants in the B domain should be interpreted with
15
16 caution before the assignment of pathogenicity. The IVS2+1G>A splicing variant was
17
18 assessed using two *in silico* splice predictors, each of which predicted the abolition of
19
20 the donor splice site. It has not been determined whether the two recurrent mutations
21
22 (His1803Arg and Leu1849Ser) arose independently or are of ancestral origin.
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29 *In silico* analysis of the five missense mutations was undertaken, utilising on –line
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31 available SIFT (<http://blocks.fhcrc.org/sift/SIFT.html>), PolyPhen
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33 (<http://genetics.bwh.harvard.edu/pph/>), PMut (<http://mmb2.pcb.ub.es:8080/PMut/>)
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35 and Russell Analysis (<http://www.russell.embl-heidelberg.de/aas/>) programs.
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37 Additionally the absence of all variants was confirmed on both ENSEMBL
38
39 (<http://www.ensembl.org/>) and Seattle SNPs (<http://pga.gs.washington.edu/>) websites.
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42 The analysis tools predict the possible impact of an amino acid substitution on protein
43
44 structure and function, based on comparisons between amino acid properties, protein
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46 homologies and evolutionary conservation. The four analysis tools gave concordant
47
48 conclusions for only 2/5 missense mutations (Gly259Glu and Leu1849Ser). For each
49
50 of the other three variants one tool gave a conflicting prediction, although in each case
51
52 the pattern of agreed results differed. These discrepancies demonstrate the limitations
53
54 of *in silico* analysis, and highlight the need for thorough examination before
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56 associating a novel variant with FV deficiency. Protein modelling was undertaken,
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3 using SwissProtDeepView software to effect the amino acid substitution and Protein
4 Explorer to visualise any local structural alterations. The native glycine at position
5
6 259 is a small residue located at the interaction between the A1 and A3 domains (fig
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8 1C). Its replacement with the large negatively charged glutamine residue (fig 1D) may
9
10 alter native domain interactions by both spatial and charge differences, as the
11
12 glutamine will prefer a buried position [28]. The function of the very large arginine
13
14 residue at position 1631 (fig 1E) may be to maintain local stability. This is likely to be
15
16 negatively affected by its substitution with the small neutral proline (fig 1F)
17
18 introducing a tight turn which produces gross conformational changes. Histidine
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20 1803 (fig 1G) is a polar residue in the A3 domain. The substitution of this residue
21
22 with arginine confers a change in charge to the region, and alters local interactions
23
24 (fig 1H). The effect of this mutation is severe, as seen in patient 9, with almost
25
26 immeasurable FV levels, where it is seen in compound heterozygosity with a
27
28 premature stop codon, encoding a null allele. The hydrophobic leucine residue at
29
30 position 1849 (fig 1I) is located in the A3 domain. Its replacement with a hydrophilic
31
32 serine residue (fig 1J) will result in conformational changes which may affect
33
34 interaction with the A1 domain. In contrast, the same amino acid substitution in the
35
36 A1 domain (Leu90Ser) is predicted to result in a limited conformational change
37
38 (figs 1A and 1B) possibly due to the relatively neutral properties of serine. The
39
40 pathogenicity of the Leu90Ser substitution must remain putative at present, but further
41
42 investigations may elicit additional insights into its function. The limited structural
43
44 damage may be suggestive of a type II mutation, with discrepant FV antigen and
45
46 activity levels, although as yet FV Ag levels have not been assessed on this patient.
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48 Expression of the serine90 variant and generation of a mutant protein would offer
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50 further support to its pathogenic function. Although the Leu90Ser mutation is the only
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3 variant identified in this patient (other than known benign polymorphisms) its co-
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5 segregation with mild FV deficiency in this family cannot be proven as reports of a
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7 bleeding tendency in previous generations in the paternal line are anecdotal. Whilst
8
9 the index case has a life long history of easy bruising and trauma associated bleeding
10
11 all her living first degree relatives are asymptomatic. Whilst extensive laboratory
12
13 investigations coupled with *in silico* analysis is generally sufficient to label a
14
15 previously unidentified variant as a mutation, caution should always be exercised in
16
17 assigning pathogenicity: the definitive tool is considered to be *in vitro* expression of
18
19 the mutant protein in cell culture. In some cases a variant may be better described as a
20
21 marker than as causative of the deficiency.
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27 The number and type of mutations reported here are broadly consistent with the
28
29 spectrum seen in other studies. Point mutations and frameshift mutations predominate,
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31 and a small number of splice variants have been detected.
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34 The number and spectrum of mutations identified within the F5 gene is increasing
35
36 rapidly as more families are studied. Each report of a novel mutation adds to our
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38 understanding of this gene, and the function of this complex protein with its dual pro-
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40 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]

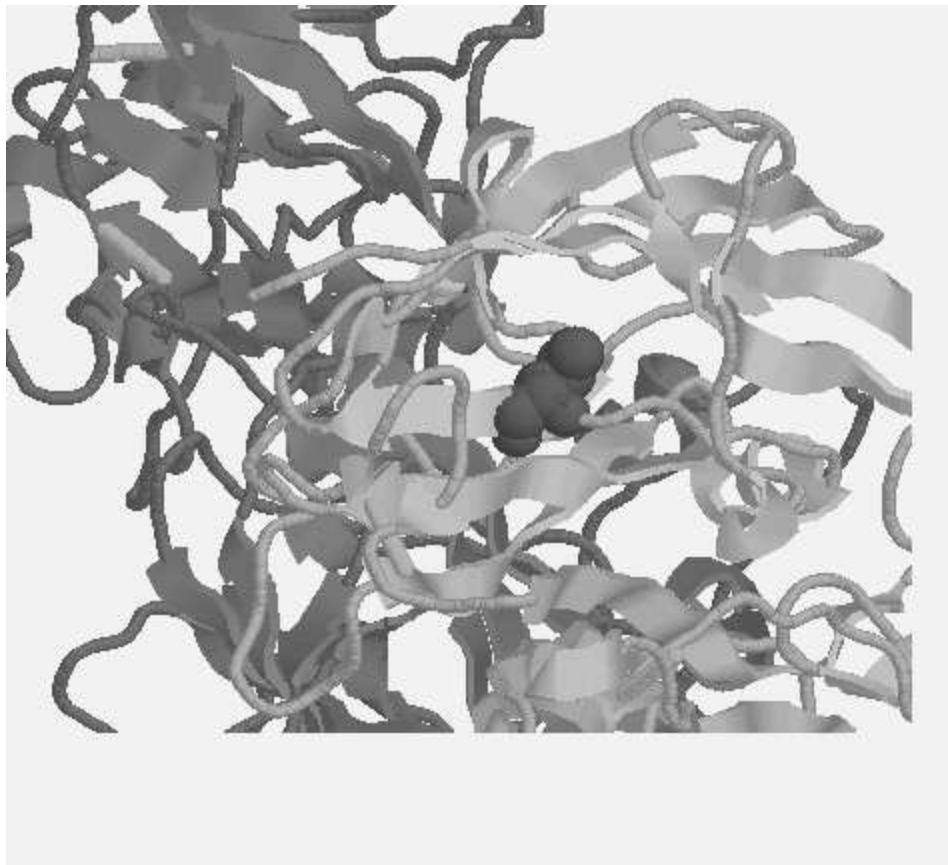
^c Normal Range 66-114 iu/dl

^d Normal Range 53-110 iu/dl

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3 **Fig 1:** Comparison of structural differences between wild type and mutant variants
4 Models were created using Protein Explorer software, with variant forms being produced
5 SwissProt Deep-View software. The models are presented as ribbon diagrams with the relevant
6 amino acid in each case being shown in 'space fill' format. The domains in each display are
7 oriented to best display the site of the mutated residue. Fig 1A+B represents Leu90Ser, 1C+D are
8 the Gly259Glu models, with the A1 and A3 domains labelled. 1E +F shows Arg1659 Pro, 1G+H
9 are the His1803Arg variants and 1I+J the Leu1849Ser. In each pairing the wild type model is
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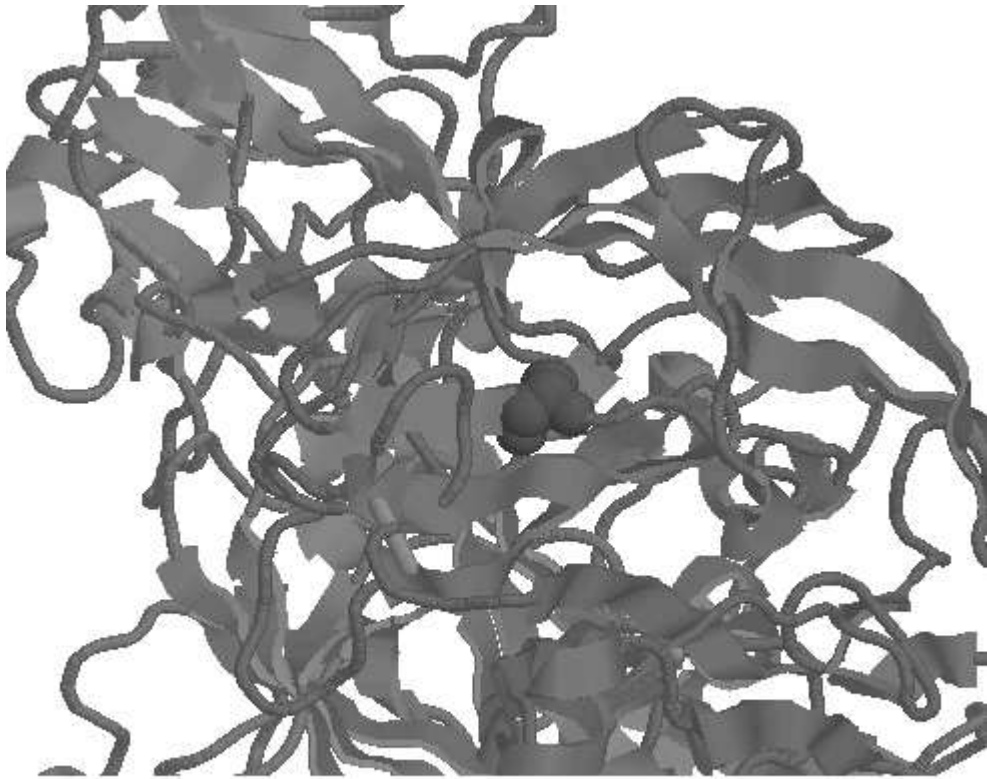
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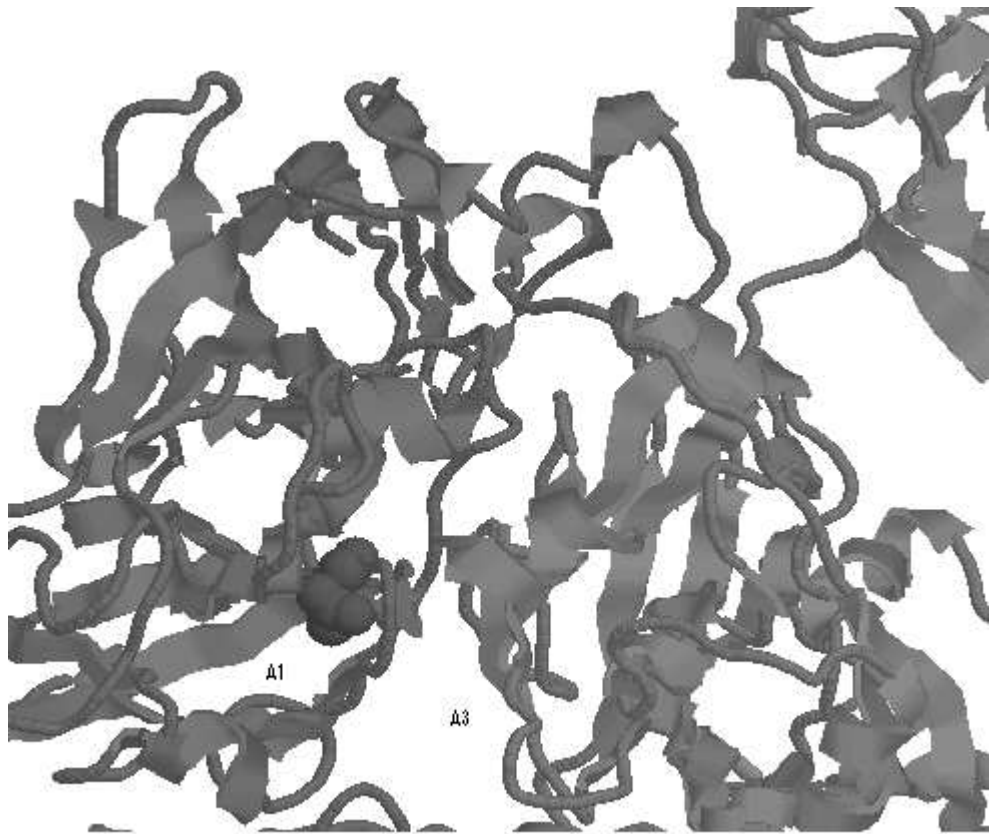


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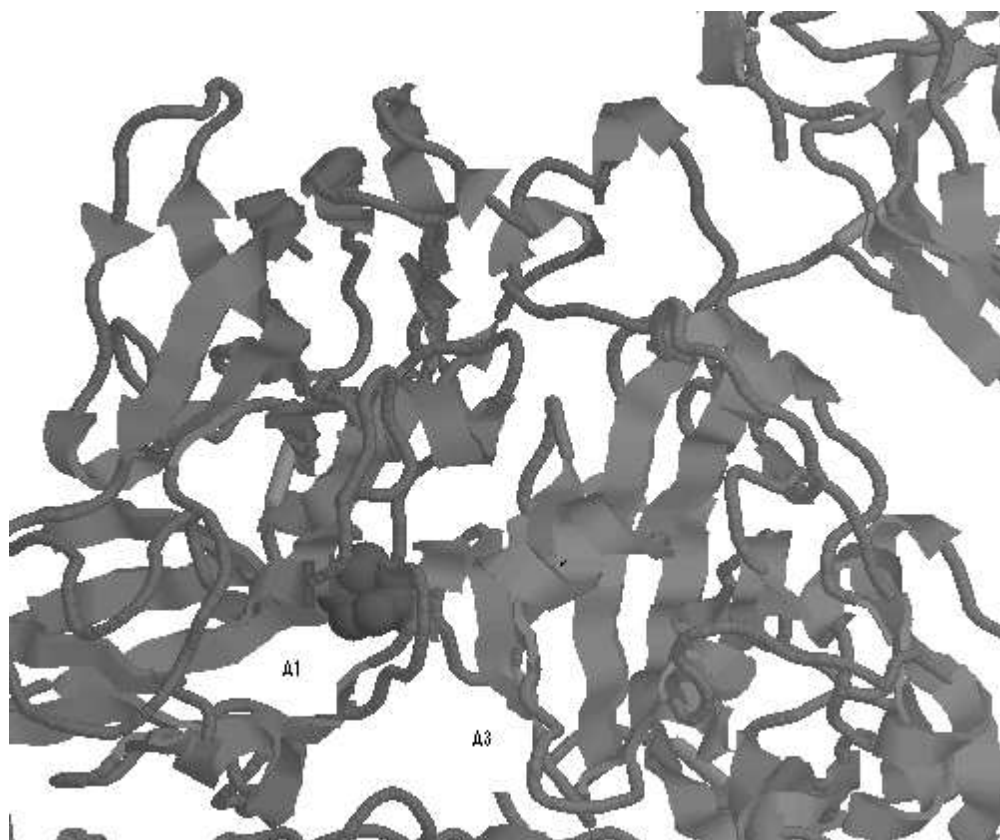
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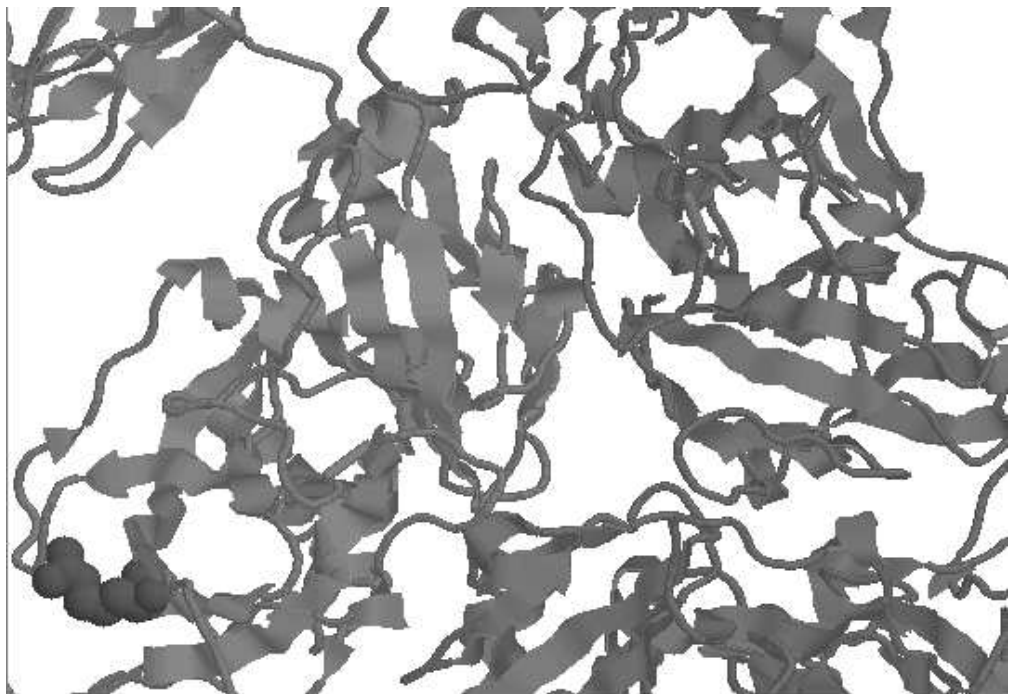


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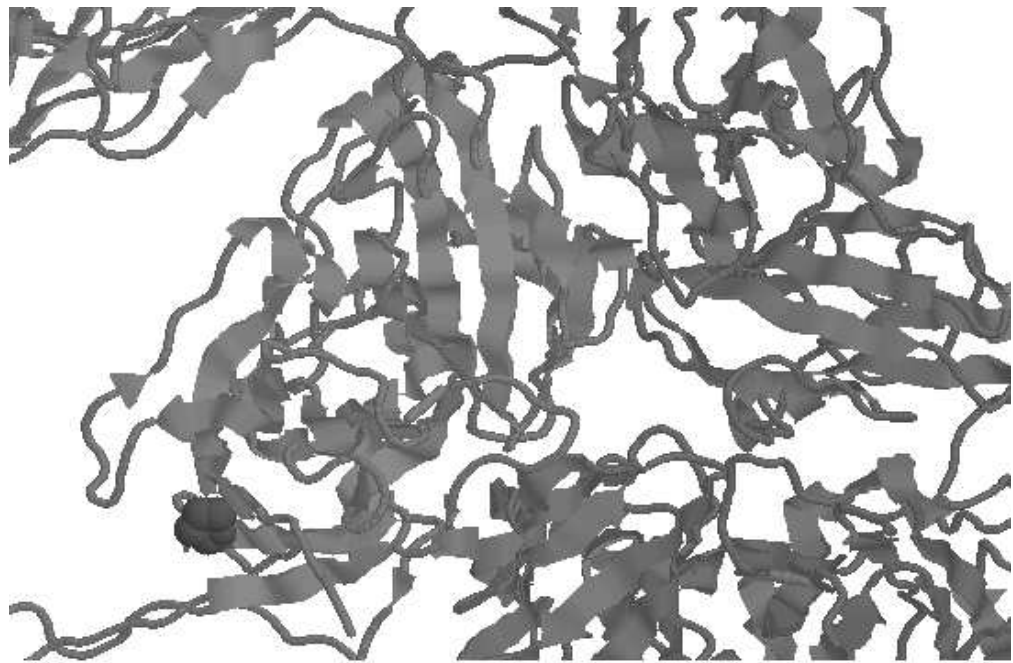
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142x113mm (96 x 96 DPI)

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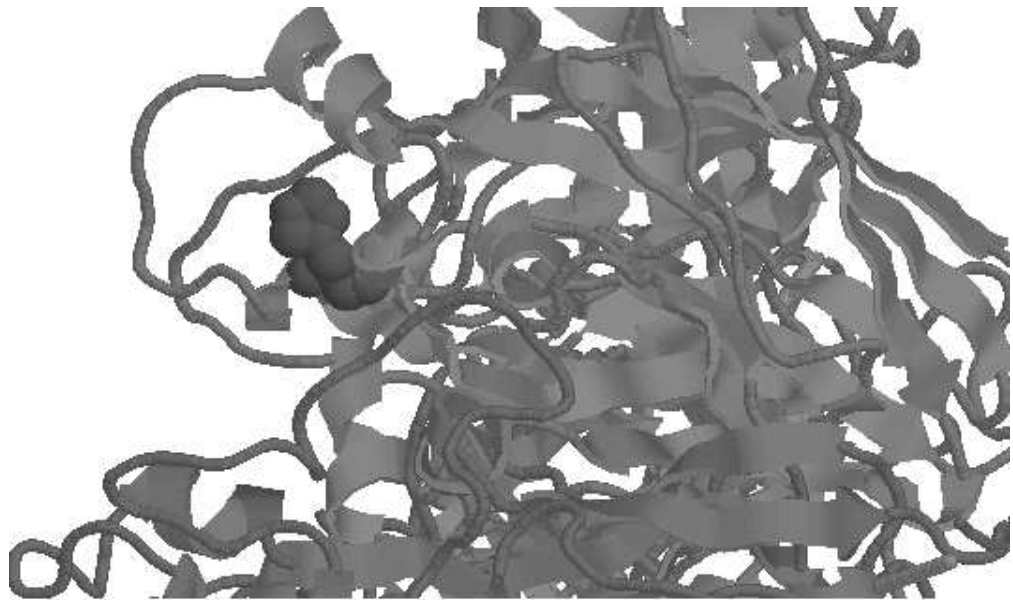
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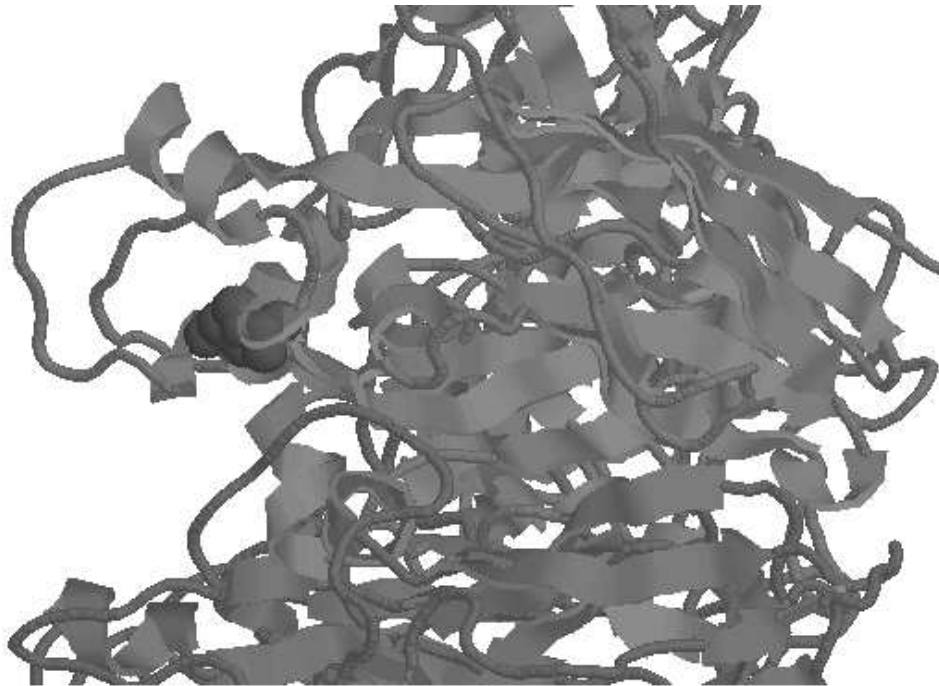
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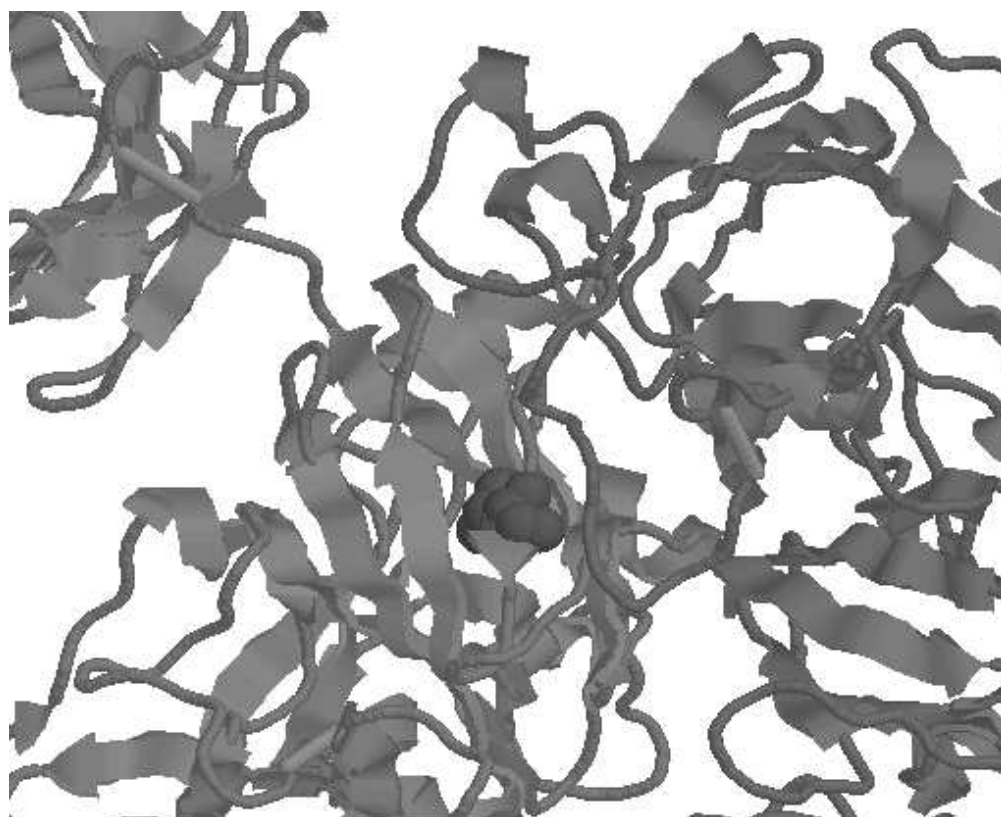
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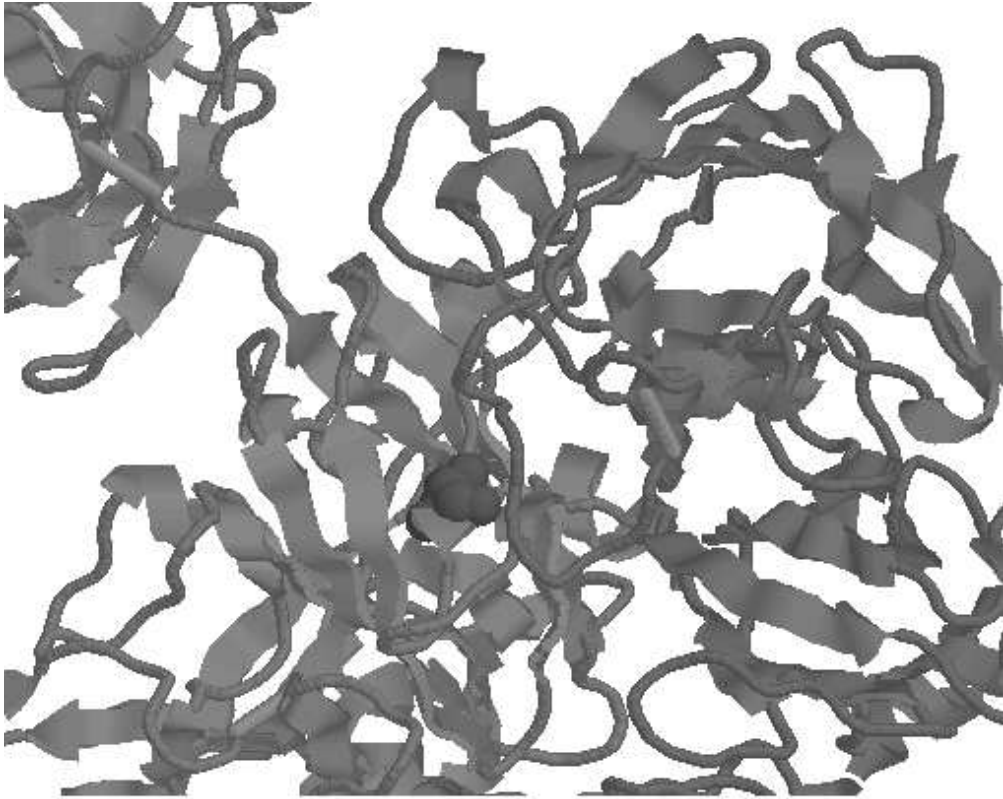
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Guy's and St Thomas' 

NHS Foundation Trust

St Thomas' Hospital

Westminster Bridge Road

London SE1 7EH

Direct line: 020 7188 2798

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

Peer Review