The natural occurrence of human fibrinogen variants disrupting inter-chain disulfide bonds (AαCys36Gly, AαCys36Arg and AαCys45Tyr) confirms the role of N-terminal Aα disulfide bonds in protein assembly and secretion

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ABSTRACT

Analyses of site-directed fibrinogen mutants expressed in several recombinant models have previously shown that both inter- and intra-chain disulfide bonds are critical for fibrinogen assembly and secretion. Four naturally occurring mutations on AαCys36 and AαCys45 residues are reported here to be associated with decreased fibrinogen levels. This confirms the main role of the AαCys36-BβCys65 and AαCys45-γCys23 disulfide bonds in reaching a normal fibrinogen plasma level. Decreased coagulant/antigen ratios indicate abnormal species secretion in heterozygous subjects which varies between individuals. However, in contrast to overexpression in experimental models, disruption of the AαCys36-BβCys65 disulfide bond did not result in the appearance of Aα-Bβ-γ moieties *in vivo*. A 188 kDa molecule reacting only with anti Aα and anti Bβ chains was found in the plasma of the AαCys45Tyr variant. Heterozygous carriers of Aα chain mutations usually have normal fibrinogen levels, in contrast to the AαCys36Gly, AαCys36Arg and AαCys45Tyr variants that are shown here to cause hypofibrinogenemia.

Key words: disulfide bond, fibrinogen, assembly, secretion.

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Introduction

Fibrinogen is a 340-kDa dimeric protein, both halves being composed of three polypeptide chains (Aα, Bβ, γ) encoded by a cluster of three genes.[1] The combination leads to a molecule organized with a central E domain connected by stranded coiled-coils to two distal D domains. The coiled-coils are in the N-terminal portion of each Aα, Bβ and γ chains. The chain assembly starts with either an Aα–γ or a Bβ–γ association, with further addition of the third chain leading to an Aα–Bβ–γ half molecule, and finally to a $(A\alpha–B\beta–\gamma)_2$ dimer. Analyses of deletions and substitutions expressed in several recombinant models have shown that these coiled-coil portions are essential for chain-chain interactions, together with 29 inter- and intra-chain disulfide bonds.[2-5] These bonds include AαCys45-γCys23 and AαCys36-BβCys65, the disruption of which can prevent formation of the (Aα–Bβ–γ)2 dimer.[4,6] However, only one naturally occurring variant has been reported so far that would confirm these data.[7,8] Three mutations of the critical AαCys36 and AαCys45 residues associated with hypofibrinogenemia are reported here together with their clinical and biological consequences. These novel variants occurring in four different families reinforce the likely role attributed to these amino acids in maintaining molecular integrity.

Design and Methods

After diagnosis, frozen plasma aliquots were centralized to perform a measurement of coagulometric fibrinogen by Clauss assay (STA coagulometer, Diagnostica Stago, Asnières, France), together with immunonephelometric measurement (Turbitimer, Dade Behring, Paris, France). Purified fibrinogen was prepared by glycine precipitation.[9] SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting of reduced and non-reduced plasma samples were performed as previously reported.[10] High-performance liquid chromatography (HPLC) was performed; fibrinogen was dissolved in Tris buffer containing urea 8 M, and run on a Vydac™ C-4 (Interchim, Montluçon, France) column.[11] In 2 patients, fibrinopeptide release was studied according to Niwa[12] and fibrin monomer repolymerization according to Brennan.[13] DNA amplification by polymerase chain reaction (PCR) and sequencing of all exons and their flanking regions of the Aα, Bβ and γ chain genes was performed with PCR primers designed with Oligo 6.0 software (Molecular Biology Insight, Cascade, CO, USA). The amplified DNA fragments were sequenced

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with the PCR primers using the dye terminator sequencing kit from Applera Courtaboeuf, France. Informed consent was obtained from all patients. This work was approved by the local ethics committees (approvals DC 2008-214 and DC 2008-880).

Results and Discussion

Fibrinogen La Seyne was found in a 47-year old woman. A blood coagulation evaluation had been performed because of the occurrence of menometrorrhagia. This bleeding feature was unique and in fact stopped after performing a hysterectomy for myofibroma. Fibrinogen was found to be decreased on several occasions, when measured both by mechanical Clauss assay and by immunonephelometry (Table 1). Liver function was normal and there were no other hemostatic defects. Release of fibrinopeptides by thrombin was normal. Sequencing of the *FGA* gene showed the presence of a heterozygous c.221T>G mutation in exon 2 predicting an AαCys36Gly mutation in the fibrinogen Aα chain.

Fibrinogen Quimper is another unrelated occurrence of the same heterozygous AαCys36Gly mutation. This was found in a 23-year old patient. The abnormal fibrinogen was noticed because of a prolonged aPTT evaluated before dental extraction. On several occasions, discrepancies existed between a decreased functional fibrinogen and a normal but low antigen or gravimetric values. The patient reported menorrhagia which was successfully suppressed by oral contraception. A family study indicated that the abnormality was likely to be inherited from her mother (fibrinogen activity = 0.85 g/L, antigen = 1.0 g/L). Her father had a normal fibrinogen (activity = 3.0 g/L, antigen = 2.2 g/L). DNA was not available from either parent. Release of fibrinopeptides A by thrombin was delayed at 5 min, as fibrinopeptide B rate was normal. Fibrin monomer repolymerization was normal.

Fibrinogen Marseilles was found in a 39-year old male who had a myocardial infarction in the absence of any risk factors. He did not report any other thrombotic or hemorrhagic events. Laboratory assays found a decreased functional fibrinogen (0.56 g/L) with borderline antigen (1.40 g/L). Sequencing found a heterozygous c.221T>C mutation in exon 2 of the Aα chain gene predicting an AαCys36Arg mutation. The same heterozygous mutation was found in his asymptomatic brother together with a decreased fibrinogen level.

For these three variants, analysis of purified fibrinogen did not show any alteration in the SDS-PAGE and HPLC profiles, and plasma immunoelectrophoresis revealed by antifibrinogen antibodies was also normal.

Fibrinogen Marseilles II was detected in a 28-year old female during routine testing performed before a tooth extraction. She reported an abnormally voluminous hematoma following a clavicle fracture. Fibrinogen (Clauss assay) was found decreased on several occasions (0.45-0.93 g/L). Tooth extraction was uncomplicated with fibrinogen prophylaxis. Sequencing of the *FGA* gene showed the presence of a heterozygous c.249G>A change in exon 3 predicting an AαCys45Tyr mutation.

Abnormal PAGE in non-reducing conditions was confirmed by immunoblot analysis of plasma, for which an additional band at 188 kDa was found (Figure 1). This band was recognized by antibodies directed against fibrinogen (polyclonal), Aα and Bβ chains (monoclonals) but not against γ chain (monoclonal) or albumin (polyclonal).

Although hampered by a low fibrinogen yield consistent with hypofibrinogenemia, HPLC was normal when testing a reduced sample. Under unreduced conditions, the main peak was hooked and a small peak eluted just after.

AαCys36 is involved in a disulfide bond with Cys65 on a Bβ chain, participating in the bridging of the AαBβγ moieties.[14] Two families presented with a Cys to Gly substitu-

Table 1. Representative biological values from the four studied individuals.

	Aα chain	Variation	*FGA* polymorphism	Aα chain polymorphism	Bβ chain	Fibrinogen plasma level Clauss	antigen	C/Ag	Clotting time thrombin	reptilase
Reference sequence		NP_068657.1	NG_008832.1 NM_021871.2	rs6050	rs4220					
	mature	HGVS nomenclature				g/L	g/L		sec	sec
Fibrinogen La Seyne	Cys36Gly	p.Cys55Gly	g.6292T>G c.221T>G	Thr312Ala heterozygous	Arg448Lys homozygous	0.41	1.05	0.39	27	19
Fibrinogen Quimper	Cys36Gly	p.Cys55Gly	g.6292T>G c.221T>G	Thr312Ala absent	Arg448Lys absent	0.95	1.50	0.63	27	14
Fibrinogen Marseille	Cys36Arg	p.Cys55Arg	g.6292T>C c.221T>C	Thr312Ala heterozygous	Arg448Lys heterozygous	0.56	1.40	0.40	30	19
Fibrinogen Marseille II	Cys45Tyr	p.Cys64Tyr	g.6780G>A c.249G>A	Thr312Ala absent	Arg448Lys absent	0.93	0.80	1.16	28	22
Control values						1.8-4.1	1.4-3.3	1.08-1.88	16-23	14-20

Functional fibrinogen is given as the electromechanical value measured in the central laboratory. Fibrinogen antigen is measured on the same sample and the ratio is calculated (C/Ag). Normal range had been determined in 490 subjects recruited by a local prevention clinic as previously reported.[4] For PCR of the FGA *gene, the primers (each 0.5 μM final) were CCCTGTGTCTGCTCTCCTTA and ATCCTGTCTGTTCACCCACT for exon 2, GAAGAGAAAGGGTAGGAA and TATTTATTTAGGATTTTTGTTG for exon 3, TAGGCAACACTTACCACTG and GTGACCTTCTCTTTACCAG for analysis of the Thr312Ala polymorphism in exon 5 (p.Thr331Ala). GAGGTCTTTGGTGTATTAGTG and GTTACTTCTTGGTGAGCAAG were used for analysis of the Arg448Lys polymorphism in exon 8 of the FGB gene (p.Arg478Lys). The conditions for amplification were 4 min at 95°C for the initial denaturation step followed by 35 cycles of denaturation at 94°C (30 sec), hybridization at 50°C (30 sec) and elongation at 72°C (1.5 min) in the presence of Gold DNA polymerase (Applera). Every patient is heterozygous for the mutation and no other mutation was found by sequencing of the three chain gene coding sequences and flanking regions with primers designed with Oligo 6.0.*

tion and another with a Cys to Arg substitution. Disruption of the AαCys36-BβCys65 disulfide bond is expected. This bond is important, as Huang *et al.* have shown that changing either AαCys36 or BβCys65 to Ser prevents formation of the (Aα–Bβ–γ)2 dimer[6] in baby hamster kidney (BHK) cells. Similarly, substitution of BβCys65 for Ser in COS-1 cells impaired hexamer assembly, and the chains were secreted in part as half molecules.[4] The data obtained from 5 patients from three families showed that AαCys36 is critical for reaching normal plasma fibrinogen levels, but the half molecule was not detectable in the plasma of studied patients. The fact that half molecule secretion is only seen in cell models may result from overexpression in the experimental model or from rapid removal of this species *in vivo*. Indeed the ratio of clotting to antigen fibrinogen levels was decreased in all 5 AαCys36 variant cases, likely reflecting secretion of abnormal molecules and hypodysfibrinogenemia. Other intermolecular links are involved in maintaining the integrity of the fibrinogen molecule, including AαCys28-AαCys28 and γCys8-γCys9. However, complete disruption of the hexamer assembly required mutations of AαCys28, BβCys65, γCys8, and γCys9 residues together.[6] Therefore, a partial interchain binding by AαCys28, γCys8 and γCys9 residues may allow to some extent the circulation of heterozygous molecules (i.e. $A\alpha A\alpha_{variant}B\beta_2\gamma_2$). The slight prolongation of thrombin clotting time may also indicate an impaired fibrinoformation tested with lower concentration of thrombin than that used for the Clauss assay, but such a prolongation has already been noticed for hypofibrinogenemia.[15] These molecular species were not detectable on the basis of their electrophoretic and chromatographic characteristics, as expected with the corresponding detection limits. These would present a unique instead of two AαCys36-BβCys65 disulfide bonds. The delay of fibrinopeptide A release indicates a variable expression of these variant molecules from one patient to another, and an impact on thrombin action. AαCys36 is located at the surface of the fibrinogen molecule, and the size of the substituting residue is not expected to influence *per se* the shape of the molecule. In contrast, the AαCys36-BβCys65 disulfide ring is located at the interface with thrombin (Figure 2A), explaining the impaired functional assay. It is likely that this disulfide bond (Figure 2B) maintains residues αPhe35 and βAla68 in the correct orientation for hydrophobic interaction with thrombin Phe34 and Tyr76, respectively (Figure 2C).[16] The data suggest that the disruption of the disulfide bond is more relevant than the nature of the substitution. In addition, for the Cys36Arg mutation, AαGlu39 and BβAsp69 salt bridges established with thrombin Lys110 and Arg77, respectively, could also be affected.

Fibrinogen Marseilles II is a confirmed hypofibrinogenemia with a heterozygous AαCys45Tyr mutation. In contrast to the AαCys36 mutations presented here, fibrinogen antigen level was decreased to a degree similar to that of the activity. Disulfide rings at the N-terminus of the coiled-coil region are BβCys76-AαCys49, BβCys80-γCys19 and AαCys45-γCys23.[5] Variants with both γCys19 and γCys23 residues changed by site-directed mutagenesis showed suppressed dimerization of the Aα–Bβ–γ moieties and intracellular accumulation of the Aα–Bβ–γ half molecules.[4] Similarly, replacement of the Aα45Cys by Phe in COS-1 cells resulted in secretion of half molecules, and has been found in a Cremona patient with hypofibrinogenemia.[7] The naturally occurring variant Marseilles II lacking the AαCys45-γCys23 disulfide bond with substitution by a Tyr similarly indicates that the existence of AαCys45 is critical to maintaining a sufficient level in plasma. Unexpected molecules were also found in plasma only under non-reducing conditions. However, unlike the AαCys45 to Phe variant, the main additional molecule had an apparent molecular weight estimated at 188 kDa. Using immunoblot analysis, it was recognized by antibodies directed against fibrinogen, Aα and Bβ chains, but not against either albumin or γ chains. This is consistent with an Aα-Bβ chain association which could occur through an unpaired Cys residue different from Aα45. Other constituents involved in the 188 kDa species are still to be identified but could also consist of a γ chain in a conformation that prevents antibody binding. This last possibility would be consistent with Redman and Xia who have shown that the Aα chain is associated after its synthesis either to a γ chain or to a Bβ-γ dimer.[5] If not, the present data would indicate that alternate assembly pathways

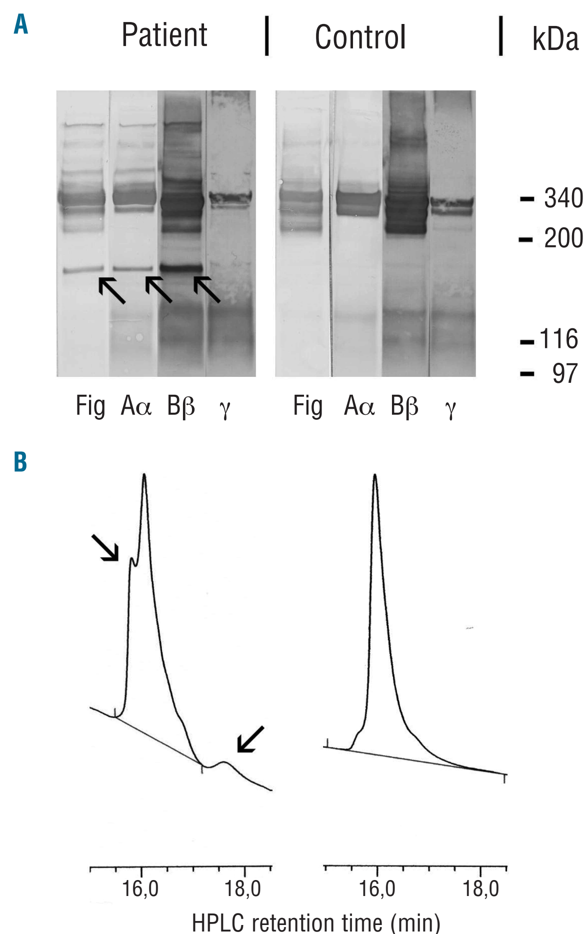

Figure 1. **Immunoblotting and HPLC of AαCys45Tyr variant in patient's plasma. (A) Immunoblotting of AαCys45Tyr mutation in patient's plasma. After non-reducing PAGE of patient and control plasma, the gel was blotted onto nitrocellulose film. Immunostaining was performed with either a polyclonal anti-fibrinogen antiserum (lanes "fig") or monoclonal antibodies against Aα, Bβ and γ chains (clones NYB1D4, NYB18C6, 2G2-H9). Bβ and γ blots have been obtained from separate migrations. Oblique arrows indicate the additional 188 kDa band. MW markers are on the right. (B) HPLC profile of the same patient's fibrinogen under non-reducing conditions. Arrows indicate the additional peaks.**

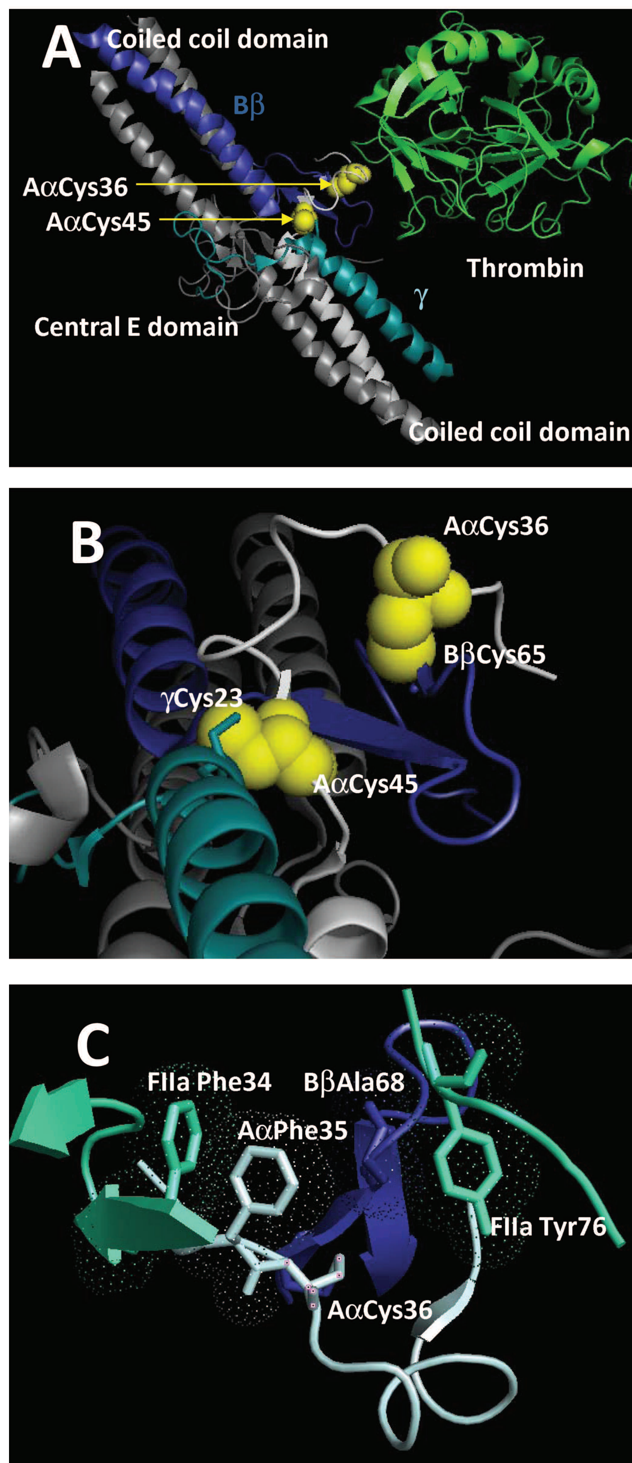

Figure 2. The AαCys36 and Cys45 residues in the central E domain of fibrinogen. The pdb file 1QVH was used to generate the figure that was drawn with PyMOL (Schrödinger, Portland, OR, USA). (A) Putative interactions of the AαCys36 and Cys45 residues with thrombin (FIIa). The AαCys36 and Cys45 residues are represented in space filled mode. Only one thrombin molecule and the relevant interacting Cys36 and Cys45 residues are shown. (B) Representation of the coiled-coil domain. The disulfide bonds involving the AαCys36, AαCys45, γCys23 and BβCys65 residues are shown. (C) Residues involved in hydrophobic interactions between thrombin (FIIa Phe34, FIIa Tyr76) and fibrinogen (AαPhe35, BβAla68) that are potentially disrupted by the AαCys36 mutations.

were used in the initial steps of the variant assembly. The Cremona and Marseille II Aα species only differ by a hydroxyl on the substituting residue. This hydroxyl appears to be critical for the assembly mechanism since for fibrinogen Cremona half molecules are assembled *in vitro*. In this regard, it would be interesting to know if abnormal species circulate in the plasma of Cremona patients. Other minor molecular species of higher molecular weight than fibrinogen existed in Marseilles II plasma and were dismantled by reduction (Figure 1). This is consistent with larger associations through unpaired Cys residues, as previously described.[17]

Most of the mutations of the *FGA* gene affecting plasma levels are detected only in afibrinogenemic patients, and heterozygous carriers have normal fibrinogen values.[18] Therefore, mutations in the *FGA* gene usually behave as recessive with very few exceptions[7,19,20] because the synthesis of the Aα chain is in excess to the other two chains. AαCys36Gly, AαCys36Arg and AαCys45Tyr variants behave as a dominant inhibitory mutant on fibrinogen secretion. With equivalent Aα chain synthesis *per* allele, random incorporation of a mutated Aα chain in the fibrinogen molecule is expected to occur in 3 out of 4 molecules. Plasma fibrinogen amounts reported here (0.41-0.95 g/L) suggest that roughly one in every 4 assembled molecules is secreted.

In conclusion, the variants reported here confirm that the AαCys36-BβCys65 and AαCys45-γCys23 disulfide bonds are critical for fibrinogen assembly and/or secretion. Contrary to overexpression in experimental models, disruption of the AαCys36-BβCys65 disulfide bond did not result in Aα-Bβ-γ moiety secretion *in vivo*, possibly because of the lack of overexpression. In contrast, decreased coagulant/antigen ratios point to abnormal species secretion, but this varies between individuals (i.e. 0.63 for fibrinogen Quimper *vs.* 0.39 for fibrinogen La Seyne, Table 1). This would possibly result from the presence of a normal Aα chain mixed with a mutant Aα chain. The coding polymorphisms AαThr312Ala and BβArg448Lys might also contribute to variations between individuals. The AαCys45 residue is also critical for secretion. However, the nature of the substituted residue in Cremona allows for the assembly of Aα–Bβ–γ moieties and impairs hexamer formation, whereas Aα–Bβ–γ moieties were not found in plasma in Marseilles II. In this case, the normal antigen/coagulant ratio and low level suggest that, apart from the 188 kDa species, only normal species are secreted.

Authorship and Disclosures

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References

1. Mosesson MW. Fibrinogen and fibrin structure and functions. J Thromb Haemost. 2005;3(8):1894-904.
2. Zhang JZ, Redman C. Fibrinogen assembly and secretion. Role of intrachain disulfide loops. J Biol Chem. 1996;271(47):30083-8.
3. Zhang JZ, Kudryk B, Redman CM. Symmetrical disulfide bonds are not necessary for assembly and secretion of human fibrinogen. J Biol Chem. 1993;268(15): 11278-82.
4. Zhang JZ, Redman CM. Role of interchain disulfide bonds on the assembly and secretion of human fibrinogen. J Biol Chem. 1994;269(1):652-8.
5. Redman CM, Xia H. Fibrinogen biosynthesis. Assembly, intracellular degradation, and association with lipid synthesis and secretion. Ann NY Acad Sci. 2001;936:480-95.
6. Huang S, Cao Z, Davie EW. The role of amino-terminal disulfide bonds in the structure and assembly of human fibrinogen. Biochem Biophys Res Commun. 1993; 190(2):488-95.
7. Platè M, Asselta R, Spena S, Spreafico M, Fagoonee S, Peyvandi F, et al. Congenital hypofibrinogenemia: characterization of two missense mutations affecting fibrinogen assembly and secretion. Blood Cells Mol Dis. 2008;41(3):292-7.
8. Hanss M, Biot F. A database for human fibrinogen variants. Ann NY Acad Sci. 2001; 936:89-90.
9. Kazal LA, Amsel S, Miller OP, Tocantins LM. The preparation and some properties of fibrinogen precipitated from human plasma by glycine. Proc Soc Exp Biol Med. 1963;113: 989-94.
10. Hanss M, Ffrench PO, Mornex JF, Chabuet M, Biot F, De Mazancourt P, et al. Two novel fibrinogen variants found in patients with pulmonary embolism and their families. J Thromb Haemost. 2003;1(6):1251-7.
11. Brennan SO, Fellowes AP, Faed JM, George PM. Hypofibrinogenemia in an individual with 2 coding (gamma82 A>G and Bbeta235 P>L) and 2 noncoding mutations. Blood. 2000;95(5):1709-13.
12. Niwa K, Takebe M, Sugo T, Kawata Y, Mimuro J, Asakura S, et al. A gamma Gly-268 to Glu substitution is responsible for impaired fibrin assembly in a homozygous dysfibrinogen Kurashiki I. Blood. 1996;87 (11):4686-94.
13. Brennan SO, Wyatt JM, Ockelford P, George PM. Defective fibrinogen polymerization associated with a novel γ279 Ala>Asp mutation. Br J Haematol. 2000; 108(2):236-40.
14. Doolittle RF, Yang Z, Mochalkin I. Crystal structure studies on fibrinogen and fibrin. Ann NY Acad Sci. 2001;936:31-43.
15. Sheen CR, Low J, Joseph J, Kotlyar E, George PM, Brennan SO. Fibrinogen Darlinghurst: hypofibrinogenaemia caused by a W253G mutation in the gamma chain in a patient with both bleeding and thrombotic complications. Thromb Haemost. 2006;96(5):685-7.
16. Pechik I, Madrazo J, Mosesson MW, Hernandez I, Gilliland GL, Medved L. Crystal structure of the complex between thrombin and the central "E" region of fibrin. Proc Natl Acad Sci USA. 2004;101 (9):2718-23.
17. Hanss M, Vergnes C, Rugeri L, Ffrench P, de Mazancourt P. A new electrophoretic variant of fibrinogen associated with venous thromboembolism, fibrinogen Bordeaux Aα Arg439>Cys. J Thromb Haemost. 2008; 6(8):1422-4.
18. Asselta R, Duga S, Tenchini ML. The molecular basis of quantitative fibrinogen disorders. J Thromb Haemost. 2006;4 (10):2115-29.
19. Hanss M, Chevreaud C, Ffrench P, Négrier C, de Mazancourt P. Clinical and biological features of 3 cases of hypofibrinogenemia associated with 3 different mutations (γ Ala341Thr, BβTyr326Cys and Aα Asp 496Asn). Thromb Haemost. 2007;98 (3):689-91.
20. Sheen CR, Brennan SO, Jabado N, George PM. Fibrinogen Montreal: a novel missense mutation (Aα D496N) associated with hypofibrinogenaemia. Thromb Haemost. 2006;96(2):231-2.