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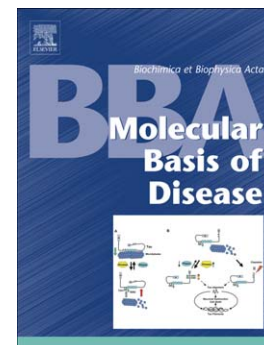
Molecular characterization of the first missense mutation in the fibrinogen Aalpha-chain gene identified in a compound heterozygous afibrinogenemic patient

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**Molecular characterization of the first missense mutation in the  
fibrinogen Aalpha-chain gene identified in a compound heterozygous  
afibrinogenemic patient**

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## SUMMARY

Congenital afibrinogenemia is a rare coagulopathy characterized by extremely low levels of functional and immunoreactive fibrinogen in plasma, associated with a hemorrhagic phenotype of variable severity. It is transmitted as an autosomal recessive trait and is invariably associated with mutations affecting 1 of the 3 fibrinogen genes (*FGA*, *FGB*, and *FGG*, coding for A $\alpha$ , B $\beta$ , and  $\gamma$  chain, respectively). Most genetic defects causing afibrinogenemia are truncating mutations, whereas only few missense mutations (6) have been identified so far, all located in *FGB*.

In this study, the mutational screening of an afibrinogenemic Italian male identified the first missense mutation (Met51Arg) in *FGA* leading to afibrinogenemia. The patient was a compound heterozygote for a previously described frameshift mutation (1215delT) in the same gene. Met51Arg involves a residue located at the very beginning of the coiled-coil domain, in a region demonstrated to play a pivotal role in hexamer formation. In-vitro expression experiments showed that Met51Arg strongly reduces secretion of hexameric fibrinogen, whereas traces of not completely assembled trimeric intermediate were found in conditioned media. Western blot analysis on the proband's plasma confirmed the presence in vivo of the trimeric fibrinogen, supporting the hypothesis that Met51Arg prevents the final step of fibrinogen assembly.

## 1. INTRODUCTION

Fibrinogen, also known as coagulation factor I, is a plasma glycoprotein with a central role in the haemostatic process both as adhesion protein essential to platelet aggregation and as precursor of insoluble fibrin that forms the haemostatic clot [1]. Moreover, as inflammation acute-phase protein, its plasma levels rise after injury and stress [2]. *FGA*, *FGB*, and *FGG* genes, clustered on chromosome 4q32.1, encode the 3 chains ( $A\alpha$ ,  $B\beta$ , and  $\gamma$ , respectively) that form fibrinogen [3]. Two sets of these 3 chains originate the 340-kDa mature hexamer, which is mainly synthesized by hepatic parenchymal cells [4] and secreted into the bloodstream. Circulating fibrinogen shows a trinodular structure in which the N-terminus of each chain contributes to constitute the central E domain, while the C-termini of the  $B\beta$  and  $\gamma$  chains form the 2 lateral globular D-domains. The E and the D domains are linked by 2 coiled-coil triple helices [5].

Fibrinogen assembly is a complex process taking place in the endoplasmic reticulum; it is thought to go through the formation of  $A\alpha$ - $\gamma$  and  $B\beta$ - $\gamma$  dimers, followed by the addition of a third chain giving rise to trimeric half molecules. These eventually dimerize originating the mature hexamer [6]. The lack of 1 chain has been demonstrated to be sufficient to prevent the assembly and secretion of hexameric fibrinogen [7]. This condition, which leads to the complete absence of clottable fibrinogen in plasma, is known as congenital afibrinogenemia, a rare disease characterized by a pattern of hemorrhagic manifestations of variable severity.

Among fibrinogen congenital abnormalities, which include dysfibrinogenemia, hypofibrinogenemia and afibrinogenemia, only congenital afibrinogenemia (Mendelian Inheritance in Man No. #202400) is inherited as an autosomal recessive trait, while

hypofibrinogenemia represents in most cases the heterozygous state of afibrinogenemia and dysfibrinogenemia is generally transmitted as a dominant disorder [8].

Among the 43 afibrinogenemia-causing mutations described thus far, most are point mutations leading to severe truncations of the corresponding chain [9]. Notably, only 3 large deletions have been described and all involve the A $\alpha$ -chain gene [10-12], while the only 6 missense mutations so far reported are all located in the B $\beta$ -chain gene (Gly347Arg, Leu353Arg, Gly400Asp, Gly414Ser, Gly434Asp, Trp437Gly) [13-17].

In this study, the characterization of the first missense mutation (Met51Arg) in the *FGA* gene leading to afibrinogenemia is reported. The mutation was identified in a male patient from Rome in compound heterozygosity with a previously identified nonsense mutation [18].

## 2. MATERIALS AND METHODS

All family members enrolled in this study signed their consent after receiving all the necessary information about the research.

### 2.1. Coagulation tests

Plasma functional fibrinogen level was measured by an assay based on fibrin polymerization time (Laboratoire Stago, Asnières, France), whose sensitivity was 5 mg/dL. An enzyme-linked immunosorbent assay (ELISA) (sensitivity: 0.0005 mg/dL), was used to measure the antigen fibrinogen level (normal ranges for both assays: 160-400 mg/dL).

### 2.2. DNA sequence analysis

Sense and antisense primers for polymerase chain reaction (PCR) amplification and full DNA sequencing of the *FGA* gene (including all introns) were designed on the basis of the sequence deposited under GenBank accession number M64982. Their sequences can be provided on request.

PCRs were performed on genomic DNA purified from peripheral blood using standard protocols. PCR products, purified by ammonium-acetate precipitation, were directly sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1 (Applied Biosystems, Foster City, CA, USA) and an ABI 3100 genetic analyzer (Applied Biosystems). Additional internal primers were used whenever necessary to complete sequencing of the amplified products. Mutation detection was performed by Factura and Sequence Navigator software packages (Applied Biosystems).

### 2.3. Site-directed mutagenesis

The mutant plasmid pRSV-Neo-A $\alpha$ -Met51Arg was obtained by site-directed mutagenesis of the pRSV-Neo-A $\alpha$  plasmid using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The mutagenic primer couple 5'-CTTCTGGCTGCAGGAGGAAAGGGTTGATTGA-3' and 5'-TCAATCAACCCTTTCCTCCTGCAGCCAGAAG-3' (underlined letters indicate the mismatch), corresponding to nucleotide positions 1752-1782 (GenBank accession number M64982) was used. The introduction of the mutation was checked by sequencing.

### 2.4. Cell cultures, transfections, and metabolic labeling

The African green monkey kidney cell line COS-1 was grown at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air, in 10-centimeter dishes (cell-seeding density: 2x10<sup>6</sup> cells/dish). Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin) and glutamine (1%) was used. Semi-confluent cells were co-transfected with equimolar quantities of pRSV-Neo-A $\alpha$ -Met51Arg (or pRSV-Neo-A $\alpha$ ), pRSV-Neo-B $\beta$ , and pRSV-Neo- $\gamma$  plasmids, using the Lipofectamine 2000<sup>TM</sup> reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer instructions. COS-1 cells were also co-transfected with equimolar amounts of pRSV-Neo-A $\alpha$  and pRSV-Neo- $\gamma$  vectors to visualize the assembly intermediate A $\alpha$ - $\gamma$  dimer, and mock transfected with the unrelated pUC18 plasmid, as negative control. Twenty-four hours after transfection, cells were washed twice with methionine- and cysteine-free DMEM (ICN Biomedicals, High Wycombe, Berks, United



Kingdom) and incubated for 2 and 16 hours in 3 mL/dish of methionine- and cysteine-free DMEM supplemented with 200  $\mu$ Ci/mL [ $^{35}$ S]-labeled methionine and cysteine (Translabel; ICN Biomedicals), 10% dialyzed fetal bovine serum, 2 mM L-glutamine, 5 mM  $\text{CaCl}_2$ , 5 mg/mL bovine serum albumin (BSA), and 0.1 mg/mL heparin.

#### *2.5. Immunoprecipitation and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)*

After metabolic labeling, conditioned media were collected in tubes containing a protease inhibitor mixture (Complete; Roche, Basel, Switzerland) and centrifuged to remove cell debris. Cells were washed 3 times with pre-chilled PBS (phosphate buffered saline) and lysed for 1 hour on ice with lysis buffer containing 1x PBS, 1% Triton X-100 and Complete. Cell lysates were centrifuged to remove cell debris. Rabbit anti-human fibrinogen antibodies (Dako, Zug, Switzerland), preadsorbed with magnetic polystyrene beads covalently bound to recombinant protein G (Dynabeads Protein G; Dynal Biotech, Oslo, Norway) at room temperature for 40 minutes, were added to cell lysates and media and incubated for 1 hour on ice. The beads were parted from the buffer using a magnet, washed 3 times with lysis buffer and resuspended in Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, and 10% glycerol). The immunoprecipitated proteins were released from protein G by boiling for 5 minutes and were resolved by non-reducing 4% or 5% and reducing 7.5% SDS-PAGE, according to Laemmli [19]. Gels were dried under vacuum at 80°C for 20 minutes. Labeled proteins were visualized by exposing gels over night to a storage phosphor screen (Amersham Pharmacia Biotech, Uppsala, Sweden) and analyzed using a Typhoon 9200 phosphor imager and the ImageQuant software (Amersham Pharmacia Biotech). In order to verify the composition of a specific band

detected by autoradiography, the corresponding radioactive area was excised from the dried gel and soaked, at room temperature, for 30 min in 0.125 M Tris-HCl, pH 6.8, containing 5% mercaptoethanol, 2% SDS, and 10% glycerol. The gel slice was carefully placed on the top of a 8.5% gel, covered with loading dye, and submitted to electrophoresis.

## 2.6. Western blot analysis

Western blot analysis on plasma was performed after resolving plasma proteins by non-reducing 6% SDS-PAGE and electroblotting polyacrylamide gels onto nitrocellulose membranes (Protran Nitrocellulose Transfer Membrane, Whatman Schleicher&Schuell, Einbeck, Germany) over night at 100 mA by means of a Biorad Trans-Blot Electrophoretic Transfer Cell (Biorad, Hercules, CA, USA). Membranes were soaked in a blocking solution containing 5% skim milk in TBST (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween20) for 1 hour at room temperature and incubated with rabbit anti-human fibrinogen antibodies (Dako) diluted 1:40,000 in blocking solution at room temperature for 2 hours. For control, non-specific rabbit purified IgG (Sigma, Saint Louis, MO, USA) were also used as primary antibody (dilution 1:5,000). Membranes were washed 3 times for 10 minutes with TBST buffer and incubated for 1 hour at room temperature with secondary horseradish-peroxidase conjugated antibodies (Envision; Dako) at a 1:5,000 dilution. After that, filters were washed 6 times for 5 minutes in TBS supplemented with 0.3% Tween, 6 times for 5 minutes in TBST, and 2 times briefly in TBS. Immunoreactive bands were detected using an enhanced chemiluminescence kit (SuperSignal West Dura, Pierce, Rockford, IL, USA) diluted 1:5, according to the manufacturer's instructions.

### 3. RESULTS

#### 3.1. Case report

The proband, a 34-years-old Italian male born in Rome from non-consanguineous asymptomatic parents, suffered from frequent post-traumatic hematomas during his life. Laboratory analysis revealed unmeasurable levels of functional fibrinogen (<5 mg/dL) and extremely reduced (1.43 mg/dL) immunoreactive fibrinogen in plasma. Both parents showed low borderline plasma antigen fibrinogen levels (182 and 161 mg/dL for the father and the mother, respectively), while the proband's sister had normal immunoreactive fibrinogen (232 mg/dL). Functional fibrinogen levels measured in the proband's parents (father: 161 mg/dL; mother: 171 mg/dL) confirmed the slight reduction observed by ELISA (Fig. 1A).

#### 3.2. Mutational screening

A previous mutational screening in this patient, performed by sequencing the whole coding regions, the exon-intron junctions and about 300 bp of the promoter regions of the *FGA*, *FGB*, and *FGG* genes had identified a single heterozygous mutation in *FGA*: 1215delT [18]. The same mutation was found in the heterozygous state in both the patient's father and sister (Fig. 1A). The second mutation contributing to the observed phenotype was expected to be localized in the same gene but could not be detected either by Southern blot analysis or by long-range PCR amplification [18]. We then decided to extend the mutational screening to the whole *FGA* sequence, seeking for a "deep intronic" mutation or for a polymorphism preventing stable annealing of 1 of the PCR primers used in the initial genetic screening and thus preventing the amplification

of the mutated allele. To this purpose, all previously sequenced *FGA* regions were newly amplified using different (exonic) primers and inspected by direct sequencing. A novel heterozygous T to G transversion, located in exon 3 of *FGA* at nucleotide position 1767 (numbering according to GenBank accession number M64982) was identified (Fig. 1B). The same genetic alteration was found in the patient's mother. Sequencing of the region recognized by the forward primer used to amplify exon 3 (*FGA*-ln2-F, see Fig. 1B) in the first mutational screening, revealed both the proband and his mother to be heterozygous for a T to C variation (rs2070016). This nucleotide substitution corresponds to the 3' end of the *FGA*-ln2-F primer, and is therefore likely to prevent the amplification of *FGA* exon 3 (Fig. 1B).

At the protein level, the 1767T>G substitution determines the introduction of an arginine (AGG) instead of a methionine (ATG) at codon 51 of the A $\alpha$  chain (numbering omitting the signal peptide). This residue is placed at the very beginning of the coiled-coil domain (Fig. 2A), in a region that is predicted to play an important role in the hexamer assembly [20]. Further supporting the possible pathogenic role of Met51Arg, it should be noted that the involved residue is located just downstream of the disulfide ring (Fig. 2B) and lies in a region highly conserved among vertebrates (Fig. 2C).

### 3.3. Expression of wild-type and mutant fibrinogen molecules

To evaluate the effects of the Met51Arg substitution on fibrinogen synthesis, assembly, stability, and secretion, wild-type and mutant fibrinogens were in-vitro expressed in COS-1 cells (not expressing fibrinogen). To this aim, the identified mutation was inserted by site-directed mutagenesis in the mammalian expression vector pRSV-Neo-A $\alpha$  to obtain the mutant construct pRSV-Neo-A $\alpha$ -Met51Arg. COS-1 were then transiently co-

transfected with equimolar quantities of pRSV-Neo-B $\beta$ , pRSV-Neo- $\gamma$ , and pRSV-Neo-A $\alpha$  or pRSV-Neo-A $\alpha$ -Met51Arg to mimic the wild-type and the mutant conditions, respectively. Moreover, considering that during the initial steps of the fibrinogen assembly the only intermediate involving the A $\alpha$  chain is the A $\alpha$ - $\gamma$  heterodimer [21], a co-transfection experiment with the pRSV-Neo-A $\alpha$  and pRSV-Neo- $\gamma$  plasmids was also set up. As negative control, COS-1 cells were also mock-transfected with the unrelated pUC18 plasmid. Transfected cells were incubated with [ $^{35}$ S]-methionine and [ $^{35}$ S]-cysteine for 2 and 16 hours. Recombinant radiolabeled fibrinogen molecules were immunoprecipitated from culture media and cell lysates and resolved by non-reducing and reducing SDS-PAGE. In cells transfected with the 3 wild-type fibrinogen plasmids, the specific wild-type 340-kDa fibrinogen band was present both in media and lysates after 2- (data not shown) and 16-hours incubation (Fig. 3A, and 3B left panel). On the contrary, in all samples from cells transfected with the pRSV-Neo-A $\alpha$ -Met51Arg the hexameric fibrinogen was undetectable, while a smaller band of about 200 kDa, probably representing an intermediate of fibrinogen assembly, was visible (Fig. 3A, and 3B left panel). The same samples run under reducing conditions showed the expected pattern of bands corresponding to the 3 fibrinogen chains (Fig. 3B, right panel), suggesting that the 200-kDa band could correspond to the trimeric fibrinogen half molecule. To confirm this hypothesis, this band was excised from the dried gel, placed in a soaking buffer (as described in Materials and Methods), and eventually re-electrophoresed on a 8.5% SDS-PAGE, under reducing conditions. As shown in figure 3C, 3 bands were resolved with molecular weights compatible with the 3 fibrinogen chains, thus demonstrating that the 200-kDa band corresponds to the trimeric fibrinogen. The additional specific fibrinogen band of about 120 kDa detected in the

lysates of cells expressing either wild-type or mutant fibrinogen (Fig. 3A) likely corresponds to  $A\alpha$ - $\gamma$  dimers, as confirmed by its presence also in cells expressing only  $A\alpha$  and  $\gamma$  chains.

#### 3.4. Western blot analysis

In order to verify whether fibrinogen half molecules were present also in the patient's plasma, Western blot analysis was performed after non-reducing 6% SDS-PAGE. A 1:500 dilution of the plasma of a wild-type individual with a normal amount of immunoreactive fibrinogen (200 mg/dL) was used as a positive control. While hexameric fibrinogen was detected both in the patient's plasma and in the positive control, the trimeric molecule was detected only in the patient's plasma (Fig. 3D).

#### 4. DISCUSSION

Only in 20% of the more than 300 cases of congenital afibrinogenemia clinically described so far the causal mutations have been identified. Most of them (95%) are point mutations giving rise to null alleles originating from nonsense, small insertions and deletions, and splicing mutations. The majority of alterations (55%) appear to be clustered in the A $\alpha$ -chain gene [9]. Despite that, all of the missense mutations causing quantitative fibrinogen deficiency (including both hypo- and afibrinogenemia) are located in the B $\beta$ - and  $\gamma$ -chain genes [9]. Very recently, a missense mutation was identified in the A $\alpha$ -chain gene in a case of hypofibrinogenemia, but its causal role was not experimentally verified [22].

A previous study from our group in a 34-years-old Italian afibrinogenemic proband born from non consanguineous parents reported the identification of a heterozygous frameshift mutation (1215delT) in *FGA* leading to the introduction of a premature stop at codon 51 (FS51stop), preceded by an abnormal sequence of 31 amino acids [18]. However, the presence of a single mutant allele was not sufficient to explain the severe phenotype of the patient, suggesting that a second unidentified mutation affecting the same gene (*FGA*) was missing [18]. Hence, we decided to screen for mutations the entire *FGA* gene. The use of different primers during re-sequencing allowed from one side the identification of the Met51Arg mutation, and from the other side to find out why this second mutation escaped the “conventional” mutational screening, i.e. the presence of a polymorphism (rs2070016) in the annealing region of the *FGA*-ln2-F PCR primer (Fig. 1B).

The newly identified Met51Arg mutation is a non-conservative amino-acid substitution affecting a residue located at the very beginning of the fibrinogen coiled-coil domain (Fig. 2). The A $\alpha$  Met51 residue is located in the centre of an inter-subunit hydrophobic core involving A $\alpha$ Pro46, A $\alpha$ Leu54,  $\gamma$ Ile25,  $\gamma$ Ala26, and  $\gamma$ Leu29. It makes van der Waals contacts with all these hydrophobic residues. Moreover, the presence of an arginine at position 51 could electrostatically repulse Arg50, inducing a conformational variation that could interfere with the dimerization of 2 trimers.

Among the different pathways of fibrinogen assembly proposed thus far [7,23], it is now accepted that the initial assembly of fibrinogen goes through the formation of A $\alpha$ - $\gamma$  and B $\beta$ - $\gamma$  heterodimers. The further addition of a third chain forms A $\alpha$ B $\beta$  $\gamma$  half molecules that dimerize to form the mature protein (A $\alpha$ B $\beta$  $\gamma$ )<sub>2</sub> [21]. The 2 half molecules are joined not only by 3 symmetrical disulfide bonds (involving A $\alpha$  Cys28,  $\gamma$  Cys8, and  $\gamma$  Cys9) and by a disulfide link between A $\alpha$ -Cys36 and B $\beta$ -Cys65 bridging the 2 half molecules, but also by the 2 disulfide rings delimiting each coiled-coil region [24]. It has been demonstrated that deletions of the N-terminus and of the first half of the coiled-coil region of the A $\alpha$  chain, including also the Met51 residue, do not impair the assembly both of A $\alpha$ - $\gamma$  and B $\beta$ - $\gamma$  dimers, and of A $\alpha$ B $\beta$  $\gamma$  half molecules, but prevent half molecules from dimerization to give rise to mature fibrinogen [20]. Site-directed mutagenesis experiments were performed by Zhang and colleagues [24] in order to disrupt the disulfide ring between cysteines 45 and 49 of the A $\alpha$  chain and the corresponding counterparts in the  $\gamma$  (Cys23) and B $\beta$  (Cys76) chains. Expression of the obtained mutants demonstrated that these cysteines are not involved in the A $\alpha$ - $\gamma$  e B $\beta$ - $\gamma$  heterodimers and A $\alpha$ B $\beta$  $\gamma$  half-molecule formation, but they are essential to the hexamer assembly process [24]. Therefore, it has



been proposed that disruption of the N-terminal disulfide ring causes the N-terminus of each chain to be distorted, thus preventing the “docking” of the 2 half molecules, and eventually the hexamer formation [24].

In this study, the pathogenic role of the Met51Arg mutation in the fibrinogen A $\alpha$  chain was explored by transient expression of the A $\alpha$ -Arg51 fibrinogen in COS-1 cells. The obtained results confirm the hypothesis put forward on the basis of the type and position of the amino-acid substitution and of literature data. In particular, the Met51Arg mutation does not seem to either affect A $\alpha$  chain synthesis or interfere with the assembly pathway, at least up to the formation of A $\alpha$ B $\beta$  $\gamma$  trimers. In fact, trimeric fibrinogen accumulated within cells expressing the mutant molecule while mature fibrinogen was extremely reduced both in cell lysates and in culture media (Fig. 3A and B). This suggests that the mutation prevents half-molecules dimerization, probably altering the formation of the disulfide bonds involving the nearby Cys49 and Cys45, essential to this final step. The presence intracellularly of similar amounts of A $\alpha$ - $\gamma$  heterodimers in cells expressing wild-type and A $\alpha$ -Arg51 fibrinogen confirms that the initial step in fibrinogen assembly involving the A $\alpha$  chain is not hampered by the mutation (Fig. 3A).

Trimeric fibrinogen was shown to be partially secreted in culture media, in agreement with the data from Xu and colleagues [20], who also found small amounts of mutant half molecules to be secreted by cells expressing N-terminal deleted A $\alpha$  chains. Consistently with these ex-vivo results, Western blot analysis performed on the proband's plasma confirmed the presence in vivo of the trimeric molecule. Unexpectedly, given the presence of a null allele (1215delT) on the other chromosome, traces of the hexameric fibrinogen were also detected in the patient's plasma, suggesting that small amounts of mature molecules can still be released by hepatocytes. The discrepancies between the

ex-vivo and in-vivo data concerning the presence of small amounts of hexameric A $\alpha$ -Arg51 fibrinogen are difficult to explain. A tentative explanation may be the existence of cell-specific differences in permissiveness for fibrinogen half-molecule dimerization and secretion.

In conclusion, the elucidation of the pathogenic role of the Met51Arg mutation provides the first experimental evidence that missense mutations affecting the fibrinogen A $\alpha$  chain can be responsible for congenital afibrinogenemia and widens our knowledge on the protein region and residues important for fibrinogen assembly.

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## REFERENCES

- [1] M.W. Mosesson, Fibrinogen structure and fibrin clot assembly, *Semin Thromb Hemost.* 24 (1998) 169-174.
- [2] I. Kushner, The acute phase response: an overview, *Methods Enzymol.* 163 (1988) 373-383.
- [3] UCSC Genome Browser, <http://genome.ucsc.edu/>, Accessed January 05, 2007, assembly March 2006.
- [4] R.F. Doolittle, Fibrinogen and fibrin, *Ann Rev Biochem.* 53 (1984) 195-229.
- [5] G. Spraggon, S.J. Everse, R.F. Doolittle, Crystal structures of fragment D from human fibrinogen and its crosslinked counterpart from fibrin, *Nature* 389 (1997) 455-462.
- [6] C.M. Redman, H. Xia, Fibrinogen biosynthesis. Assembly, intracellular degradation, and association with lipid synthesis and secretion, *Ann N Y Acad Sci.* 936 (2001) 480-495.
- [7] S.N. Roy, R. Procyk, B.J. Kudryk, C.M. Redman, Assembly and secretion of recombinant human fibrinogen, *J Biol Chem.* 266 (1991) 4758-4763.

- [8] F. Peyvandi, S. Duga, S. Akhavan, P.M. Mannucci, Rare coagulation deficiencies, *Haemophilia* 8 (2002) 308-321.
- [9] R. Asselta, S. Duga, M.L. Tenchini, The molecular basis of quantitative fibrinogen disorders, *J Thromb Haemost*, 4 (2006) 2115-2129.
- [10] M. Neerman-Arbez, A. Honsberger, S.E. Antonarakis, M.A. Morris, Deletion of the fibrinogen alpha-chain gene (FGA) causes congenital afibrinogenemia, *J Clin Invest*. 103 (1999) 215-218.
- [11] K. Watanabe, A. Shibuya, E. Ishii, M. Kurihara, S. Inoue, M. Ono, Y. Wada, M. Wakiyama, M. Zaitzu, H. Iida, K. Muraoka, S. Kinoshita, N. Hamasaki, Identification of simultaneous mutation of fibrinogen  $\alpha$  chain and protein C genes in a Japanese kindred, *Br J Haematol*, 120 (2003) 101-108.
- [12] S. Spena, S. Duga, R. Asselta, F. Peyvandi, C. Mahasandana, M. Malcovati, M.L. Tenchini, Congenital afibrinogenemia caused by uniparental isodisomy of chromosome 4 containing a novel 15-kb deletion involving fibrinogen Aalpha-chain gene, *Eur J Hum Genet*. 12 (2004) 891-898.
- [13] Y. Fang, H.L. Wang, X.F. Wang, Q.H. Fu, W.B. Wang, S. Xie, R.F. Zhou, J. Dai, Z.Y. Wang, Inherited afibrinogenemia caused by compound heterozygous mutations in the beta beta-chain of fibrinogen, *Zhongguo Shi Yan Xue Ye Xue Za Zhi*. 13 (2005) 1086-1089.

- [14] S. Duga, R. Asselta, E. Santagostino, S. Zeinali, T. Simonc, M. Malcovati, P.M. Mannucci, M.L. Tenchini, Missense mutations in the human beta fibrinogen gene cause congenital afibrinogenemia by impairing fibrinogen secretion, *Blood* 95 (2000) 1336-1341.
- [15] D. Vu, P.H. Bolton-Maggs, J.R. Parr, M.A. Morris, P. de Moerloose, M. Neerman-Arbez, Congenital afibrinogenemia: identification and expression of a missense mutation in FGB impairing fibrinogen secretion, *Blood* 102 (2003) 4413-4415.
- [16] L. Monaldini, R. Asselta, S. Duga, F. Peyvandi, K. Ghosh, M. Malcovati, M.L. Tenchini, Fibrinogen Mumbai: intracellular retention due to a novel G434D mutation in the B $\beta$ -chain gene, *Haematologica* 91 (2006) 628-633.
- [17] S. Spena, R. Asselta, S. Duga, M. Malcovati, F. Peyvandi, P.M. Mannucci, M.L. Tenchini, Congenital afibrinogenemia: intracellular retention of fibrinogen due to a novel W437G mutation in the fibrinogen Bbeta-chain gene, *Biochim Biophys Acta* 1639 (2003) 87-94.
- [18] R. Asselta, S. Duga, S. Spena, E. Santagostino, F. Peyvandi, G. Piseddu, R. Targhetta, M. Malcovati, P.M. Mannucci, M.L. Tenchini, Congenital afibrinogenemia: mutations leading to premature termination codons in fibrinogen

$A\alpha$ -chain gene are not associated with the decay of the mutant mRNAs, Blood 98 (2001) 3685-3692.

- [19] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680-685.
- [20] W. Xu, D.W. Chung, E.W. Davie, The assembly of human fibrinogen. The role of the amino-terminal and coiled-coil regions of the three chains in the formation of the alphas $\gamma$  and betas $\gamma$  heterodimers and alphas $\beta$ gamma half-molecules, J Biol Chem. 271 (1996) 27948-27953.
- [21] S. Huang, E.R. Mulvihill, D.H. Farrell, D.W. Chung, E.W. Davie, Biosynthesis of human fibrinogen. Subunit interactions and potential intermediates in the assembly, J Biol Chem. 268 (1993) 8919-8926.
- [22] C.R. Sheen, S.O. Brennan, N. Jandolo, P.M. George, Fibrinogen Montreal: a novel missense mutation ( $A\alpha$  D496N) associated with hypofibrinogenaemia, Thromb Haemost. 96 (2006) 231-232.
- [23] R. Hartwig, K.J. Danishefsky, Studies on the assembly and secretion of fibrinogen, J Biol Chem. 266 (1991) 6578-6585.
- [24] J.Z. Zhang and C.M. Redman, Role of interchain disulfide bonds on the assembly and secretion of human fibrinogen, J Biol Chem. 269 (1994) 652-658.

## FIGURE LEGENDS

**Figure 1:** Pedigree of the analyzed afibrinogenemic family and identification of 1215delT and 1767T>G mutations in *FGA*.

**(A)** Pedigree of the analyzed family. Functional and immunoreactive plasma fibrinogen levels (mg/dL), as well as the identified mutations are reported below each symbol. The proband is indicated by an arrow.

**(B)** Strategies adopted to identify the 2 mutations. The top and the bottom panels show a schematic representation of part of *FGA* (from the promoter region to intron 3; exons are represented by boxes, introns by lines, and are not drawn to scale). The primers used to amplify exons 2 and 3 are indicated by arrows. M1 and M2 indicate the position of the 2 point mutations (1767T>G and 1215delT, respectively). The asterisk on allele 1 indicates the polymorphic site preventing amplification with the primer couple *FGA*-In2-F/*FGA*-In3-R. The middle panel shows electropherograms of the region surrounding the identified mutations in the  $A\alpha$ -chain gene. The position of the mutations (numbered according to GenBank, accession number M64982) is indicated by an arrow. K denotes T or G nucleotides.

**Figure 2:** Position of the Met51Arg mutation in the fibrinogen  $A\alpha$ -chain structure.

**(A)** Ribbon diagram of human fibrinogen E domain and N-terminal portion of coiled-coil regions, produced using the SwissPDB 3.7 software and the coordinates deposited under Protein Data Bank entry “2A45”. The position of Met51 in the  $A\alpha$  chain is indicated. The model is oriented to best display the sites of mutated residues. The N- and C-terminal residues of each chain are also indicated.



**(B)** Amino-acid sequence of the N-terminal portion of the coiled-coil region (25 residues of each fibrinogen chain are reported). The 6 cysteines involved in the 3 interchain disulfide bonds responsible for holding the molecule together (known as N-terminal disulfide ring) are boxed in gray. The Met51 residue is pointed by an arrowhead.

**(C)** Multiple alignment of the first 25 residues of the fibrinogen A $\alpha$  chain belonging to the coiled-coil region from several vertebrates. Protein sequences were obtained from Ensembl ([www.ensembl.org](http://www.ensembl.org)) under the accession numbers P02671 (*H. sapiens*), NP\_034326 (*M. musculus*), P06399 (*R. norvegicus*), P02672 (*B. taurus*), ENSMODG00000001252 (*M. domestica*), P14448 (*G. gallus*), ENSXETG00000026093 (*X. tropicalis*), NP\_001002039 (*D. rerio*), and NEWSINFRUG00000153940 (*T. rubripes*). Protein numbering omits the signal peptide. The length of the signal peptides, when not available in the literature, has been predicted using the CBS (Center for Biological Sequence analysis) SignalP 3.0 Server (<http://www.cbs.dtu.dk/services/SignalP/>). The arrowhead indicates the position of the Met51 residue. Conserved residues are shaded in grey, while identical amino acids are in bold.

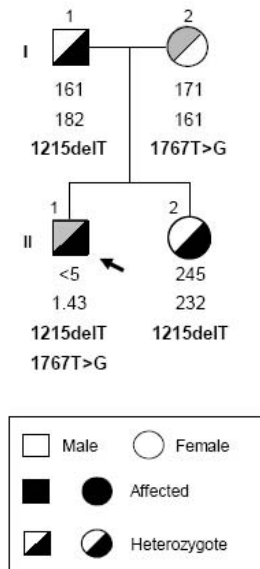
**Figure 3:** SDS-PAGE analysis of wild-type and Met51Arg mutant fibrinogens and Western blot on the patient's plasma.

Immunoprecipitated proteins from cell lysates **(A)** and conditioned media **(B)** of COS-1 cells transfected with equimolar mixtures of wild-type B $\beta$  and  $\gamma$  plasmids, together with the wild-type or the Met51Arg A $\alpha$  construct. Cells were also transfected with the wild-type A $\alpha$  and  $\gamma$  plasmids; mock transfected (pUC18) COS-1 cells represent the negative control. Cells were labeled with [ $^{35}$ S]-methionine and [ $^{35}$ S]-cysteine. Samples were separated on 5% (panel A) and 4% (panel B, left) SDS-PAGE under non-reducing

conditions, as well as on 7.5% SDS-PAGE (panel B, right) under reducing conditions. The boxed band (panel B, left) was excised from the gel, and run again on a 8.5% gel under reducing conditions (panel C).

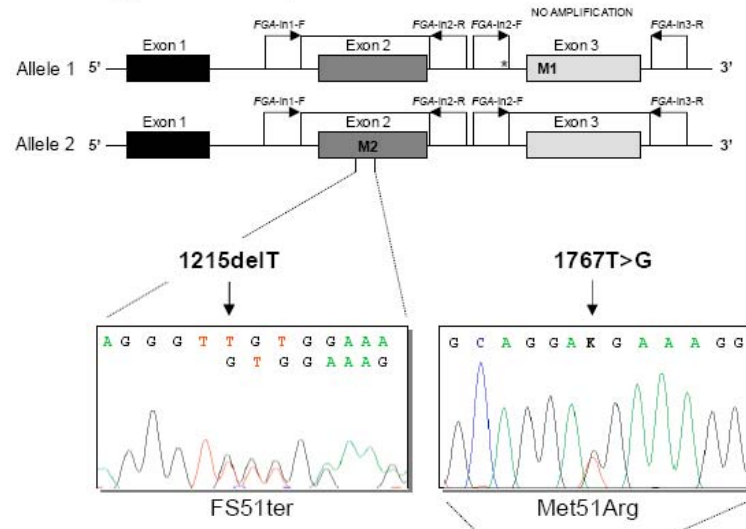
(D) Western blot analysis of the plasma of the patient and of a healthy control individual. Proteins were separated on a 6% SDS-PAGE under non-reducing conditions. The patient's plasma was loaded as such, whereas the plasma of the healthy control individual (having an immunoreactive fibrinogen level of 200 mg/dL) was diluted 1:500. The lane "proband (IgG)" contains a control sample in which the patient's plasma was loaded as such and the primary antibody was replaced by a non-specific IgG. The trimeric fibrinogen band is indicated by an arrowhead.

**A**

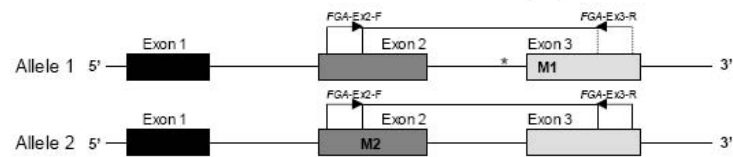


**B**

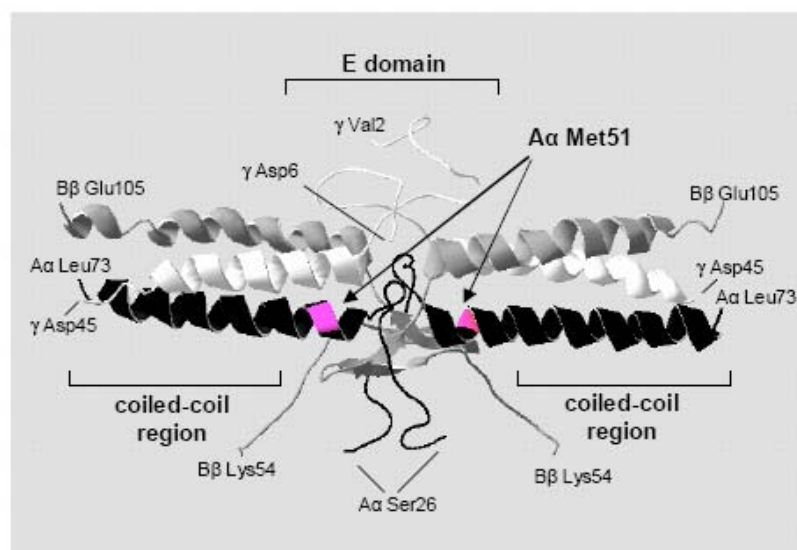
"Old" strategy [Asselta *et al.*, 2001]



"New" strategy [this paper]



**A**



**B**



**C**

