# Fibrinogen Mumbai: intracellular retention due to a novel G434D mutation in the Bβ-chain gene

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Background and Objectives. Afibrinogenemia and hypofibrinogenemia are rare inherited coagulation disorders characterized by hemorrhagic manifestations of variable entity and by plasma fibrinogen deficiency. So far, 57 mutations have been associated with these disorders, and 18 of these are missense mutations. The aim of this study was to characterize the molecular mechanism underlying severe hypofibrinogenemia in a proband from India.

Design and Methods. The mutational screening was accomplished by DNA sequencing of the three fibrinogen genes. The mutant protein was expressed in COS-1 cells, and intracellular and secreted mutant fibrinogen was analyzed by means of pulse-chase experiments.

Results. A novel homozygous G→A transition in exon 8 (nucleotide position 8017) was found in the proband's fibrinogen Bβ-chain gene. The resulting G434D missense mutation (fibrinogen Mumbai) involves a highly conserved amino acid residue, located in the C-terminal globular D domain. *In vitro* expression experiments demonstrated intracellular retention of the mutant fibrinogen and marked reduction of its secretion.

Interpretation and Conclusions. The G434D substitution causes severe hypofibrinogenemia by impairing fibrinogen secretion. Expression data confirm the importance of Bβ-chain D domain folding in the intracellular processing of fibrinogen.

Key words: congenital afibrinogenemia, hypofibrinogenemia, fibrinogen Bβ chain, missense mutation, protein *in vitro* expression.

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Fibrinogen is a 340-kDa plasma glycoprotein converted into insoluble fibrin during the last step of the coagulation process by thrombin proteolytic cleavage.[1,2] The fibrinogen molecule is synthesized and secreted into the bloodstream by hepatic parenchymal cells as a hexamer composed of two identical heterotrimers, each consisting of one Aα, one Bβ, and one γ chain.[1] The three genes (*FGA, FGB*, and *FGG*) coding for the fibrinogen chains (Aα, Bβ, and γ, respectively) are clustered in a 50-kb region on chromosome 4 (4q31.3)[3] and their co-ordinated transcription is up-regulated in response to various stress stimuli, such as inflammation and tissue injury.[4] Fibrinogen secretion requires the protein's step-wise assembly, which includes the formation of an extensive network of both inter- and intra-chain disulphide bonds.[5] The 450-Å-long trinodular structure of the fibrinogen molecule is composed of two symmetrical distal globular D domains (constituted by the C-termini of the Bβ and γ chains) joined to a central E domain (consisting of the N-termini of the six chains) by coiled-coil regions.[6,7] Traditionally, inherited fibrinogen abnormalities are classified on the basis of the plasma concentration of the protein into type I deficiencies (hypofibrinogenemia and afibrinogenemia) with reduced or unmeasurable antigen and functional levels, and type II deficiencies (dysfibrinogenemia and hypo-dysfibrinogenemia) with normal or altered antigen levels associated with reduced coagulant activity. Type I deficiencies (OMIM #202400, +134820, *134830, *134850) are rare autosomal disorders characterized by bleeding manifestations ranging from mild to severe and by the complete absence or reduced levels of fibrinogen.[8] The hemorrhagic diathesis in afibrinogenemic patients is frequently manifested as umbilical cord bleeding, hemarthrosis, menorrhagia, and post-partum bleeding; whereas hypofibrinogenemic patients usually experience milder symptoms.[8] Moreover, some afibrinogenemic patients show thrombotic complications.[9] Afibrinogenemia and hypofibrinogenemia have been traditionally considered as two distinct clinical entities, but they actually represent the same disorders, being the phenotypic expression of the homozygous or heterozygous condition for mutations causing defects in fibrinogen synthesis, assembly or secretion. All the 57 mutations causing type I deficiency so-far reported are located within the fibrinogen gene cluster.[10-25] Seventy percent of them are predicted to cause the synthesis of C-terminal truncated polypeptides, not competent for secretion. Among mutations occurring in the *FGB* gene, ten are truncating mutations (three splicing defects, six nonsense, one frameshift) and seven are missense, most of them being located within the globular D

domain of the fibrinogen Bβ chain.[16,17,26-28]

In this paper, we report the identification and the molecular characterization of a novel missense mutation (G434D or fibrinogen Mumbai) in the Bβ-chain gene of a patient from India suffering from severe hypofibrinogenemia. The role of this mutation was investigated by expression experiments of the mutant fibrinogen molecule in COS-1 cells, demonstrating that G434D impairs fibrinogen secretion.

## Design and Methods

This study was approved by the Institutional Review Board of the University of Milan. Citrate-anticoagulated blood samples were withdrawn from the patient after acquiring appropriate informed consent.

### Coagulation tests

Plasma functional and antigen fibrinogen levels were measured by an assay based on fibrin polymerization time (Laboratoire Stago, Asnières, France) and by an enzyme-linked immunosorbent assay (ELISA),[29] respectively. The sensitivities of the functional and immunologic assays were 5 and 0.0005 mg/dL, respectively (normal values for both tests ranged between 160 and 400 mg/dL).

### DNA sequence analysis

Genomic DNA was extracted from whole blood using the PUREGENE Kit (Gentra Systems, Minneapolis, MN, USA), following the manufacturer's instructions. Polymerase chain reaction (PCR) amplifications were performed on genomic DNA under standard conditions in a PTC-100 thermal cycler (MJ-Research, Watertown, MA, USA). Oligonucleotides (purchased from Invitrogen, Carlsbad, CA, USA) were designed on the basis of known sequences of the three fibrinogen genes and intergenic regions (GenBank accession numbers M64982, M64983, M10014, U36478, and AF229198). PCR conditions and primer sequences are available on request. The amplified fragments were purified by ammonium acetate precipitation and directly sequenced on both strands using the BigDye Terminator Cycle Sequencing v3.1 Kit (Applied Biosystems, Foster City, CA, USA). Sequencing primers were the same as the ones used in the amplification reactions except those used for the long exon 5 of the Aα-chain gene, which was sequenced using additional internal primers. Sequencing products were analyzed on an ABI-3100 DNA sequencer (Applied Biosystems). Factura and Sequence navigator software (Applied Biosystems) were used for mutation detection.

### Site-directed mutagenesis

The pRSV-Neo-B, plasmid, containing the full-length cDNA coding for the fibrinogen Bβ chain,[30] was used as the template for site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), according to the protocol provided with the kit. The mutagenic primer couple 5'-ATGGCACAGATGATGATGTAGTATGGATGAA-3' and 5'-TTCATCCATACTACATCATCATCTGTGC-CAT-3' (underlined letters indicate the mismatch), corresponding to nucleotide positions 8002-8032 (GenBank accession number M64983), was used. The resulting mutant plasmid (pRSV-Neo-Bβ-G434D) was checked by sequencing.

### Cell cultures, transfections, and metabolic labeling

Human hepatoma HepG2 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) and Ham's F12 media (1:1 vol/vol), supplemented with 10% fetal calf serum. The African green monkey kidney COS-1 cell line was maintained in DMEM containing 10% fetal calf serum. Glutamine (1%) and antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin) were added to both media. Cells were grown at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air, according to standard procedures. Semiconfluent COS-1 cells were transfected with either the wild-type or mutant pRSV-Neo-Bβ plasmid, together with equimolar amounts of pRSV-Neo-Aα and pRSV-Neo-γ vectors, containing the cDNA encoding the Aα and γ chain, respectively.[31] As a negative control, COS-1 cells were also mock transfected with the unrelated pUC18 plasmid. All plasmids were extracted using the EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany). Cells were transfected using the Lipofectamine 2000™ reagent (Invitrogen), following the manufacturer's instructions. For immunoprecipitation analyses, transfections were performed in six-well plates. Thirty-six hours after transfection, cells were washed twice with methionine- and cysteine-free DMEM (ICN Biomedicals, High Wycombe, Berks, UK) and incubated in 1.5 mL/well of methionine- and cysteine-free DMEM supplemented with 200 μCi [$^{35}$S]-labeled methionine and cysteine (Translable; ICN Biomedicals), 10% dialyzed fetal calf serum, 2 mM L-glutamine, 2.5 mM $CaCl_2$, 5 mg/mL bovine serum albumin, and 0.1 mg/mL heparin. After labeling for 2-hours, cells were washed twice with phosphate-buffered saline (PBS) and chasing was performed for various time periods (0, 1, 2, 4, and 8 hours) by adding 1 mL/well of DMEM supplemented with a 10-fold excess of cold methionine and a 5-fold excess of cold cysteine, 10% fetal calf serum, 2 mM L-glutamine, 2.5 mM $CaCl_2$, 5 mg/mL bovine serum albumin, and 0.1 mg/mL heparin.

### Immunoprecipitation, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and endoglycosidase-H treatment

Preparation of cell lysates and conditioned media, and immunoprecipitations were performed as previously described.[16] The immunoprecipitated proteins were released from protein A by boiling for 5 min in SDS-PAGE non-reducing Laemmli loading buffer[32] and were resolved by 4% SDS-PAGE under non-reducing conditions. Gels were dried under vacuum at 80°C for 1 hour. Labeled proteins were visualized by exposing gels overnight to a storage phosphor screen (Amersham Pharmacia Biotech, Uppsala, Sweden) and analyzed

using a Typhoon 9200 phosphor imager (Amersham Pharmacia Biotech). Endoglycosidase-H (endo-H) treatment was carried out as described elsewhere.[33]

## Results

### Clinical details of the proband

The analyzed proband was a woman from India born from a first-degree consanguineous marriage. The patient suffered from prolonged umbilical cord bleeding at birth; subsequently, her symptoms were an increased tendency to bleed in response to light traumas, and menorrhagia. The proband died due to a cerebral hemorrhage at the age of 25, a few months before the completion of the molecular characterization reported here. The proband's fibrinogen plasma levels were 35 mg/dL (antigen) and 39 mg/dL (functional). However, these values could be overestimates since the patient received a whole-blood transfusion 12 days before the fibrinogen measurements. The proband's 22-year old brother and 19-year old sister are asymptomatic. Unfortunately, no biological samples were available from any of the proband's relatives.

### Identification of the G434D mutation

The proband's fibrinogen chain genes were subjected to direct sequencing as previously described.[26] A novel homozygous transition (G to A), located in exon 8 of the fibrinogen Bβ-chain gene at nucleotide position 8017 (numbering according to GenBank accession number M64983), was the only nucleotide variation identified. This genetic defect results in a novel missense mutation, from glycine (GGT) to aspartate (GAT) at codon 434 (numbering omitting the signal peptide). Gly434 is a highly conserved amino acid residue localized in the C-terminal globular D domain of the Bβ chain[6] (Figure 1A-B).

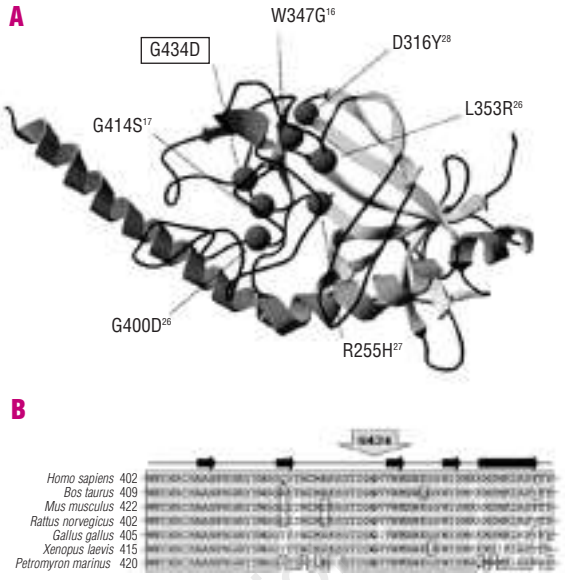


**Figure 1. Localization of the G434D mutation within the fibrinogen Bβ-chain structure. A. Ribbon diagram of the fibrinogen Bβ-chain C-terminal domain produced using the DeepView/Swiss-PdbViewer v3.7 software[34] and the PDB (Protein Data Bank) 1FZA entry.[6] The four missense mutations so far reported as responsible for congenital afibrinogenemia (L353R,[26] G400D,[26] G414S,[17] and W437G[16]) and the two Bβ-chain missense mutations responsible for hypofibrinogenemia (R255H[27] and D316Y[28]) are indicated. The novel missense mutation characterized in this paper (G434D) is boxed. An appropriate reference is given for each mutation. B. Multiple alignment of the C-terminal regions of fibrinogen Bβ chains from humans, cattle, mouse, rat, chicken, xenopus, and lamprey. Protein sequences were obtained from the Swiss-Prot database (*http://www.expasy.org/sprot/*) under the accession numbers P02675, P02676, Q8K0E8, Q02020, P14480, Q091589, and P02678, respectively. Residues are numbered omitting the signal peptide. Conserved amino acids are boxed, identical residues are shaded in gray, and the highly conserved mutant residue Gly434 is in bold type. Black arrows highlight β-strands and refer to the human protein.**

### Analysis of wild-type and mutant fibrinogen molecules

The effects of the G434D substitution on fibrinogen synthesis, stability, and secretion, were investigated by *in vitro* expression of the mutant fibrinogen molecule using the pRSV-Neo-Aα, pRSV-Neo-Bβ, and pRSV-Neo-γ mammalian expression vectors. The identified mutation was inserted into the pRSV-Neo-Bβ vector by site-directed mutagenesis, to produce the mutant pRSV-Neo-Bβ-G434D construct. Wild-type and mutant homozygous phenotypes were mimicked by transiently co-transfecting COS-1 cells (not expressing fibrinogen). A 2-hour pulse with [$^{35}$S]-labeled methionine and cysteine was followed by various chase periods (0, 1, 2, 4, and 8 hours). Recombinant fibrinogen molecules were collected by immunoprecipitation from radiolabeled media and cell lysates after each chase period. As for the wild-type protein, SDS-PAGE under non-reducing conditions showed, as expected, the specific 340-kDa fibrinogen band decreasing in intensity in cell lysates and increasing in culture media (from 1 to 8 hours of chase), concordant with normal secretion of the protein (Figure 2A,B, left). By contrast, the G434D mutant fibrinogen was always detected intracellularly but it was undetectable in conditioned media at the corresponding periods of chase, suggesting impaired secretion of the mutant molecule (Figure 2A,B, right). Positive and negative controls (fibrinogen-expressing HepG2 cells and pUC18-transfected COS-1 cells, respectively) gave the expected results (Figure 2A,B). Endoglycosidase H treatment was used to evaluate translocation of the G434D mutant protein from the endoplasmic reticulum to the *trans*-Golgi compartment.[35] Endoglycosidase-H cleaves high-mannose N-linked oligosaccharides (typical of proteins found in the endoplasmic reticulum and *cis*-Golgi compartments) but does not affect complex oligosaccharides (typical of *trans*-Golgi proteins). Immunoprecipitated wild-type and G434D mutant fibrinogens from cell lysates were treated with endoglycosidase-H and analyzed by SDS–PAGE under reducing conditions. Both mutant and wild-type Bβ and γ chains showed a shift in electrophoretic mobility (*data not shown*), suggesting sensitivity to the endoglycosidase-H treatment.

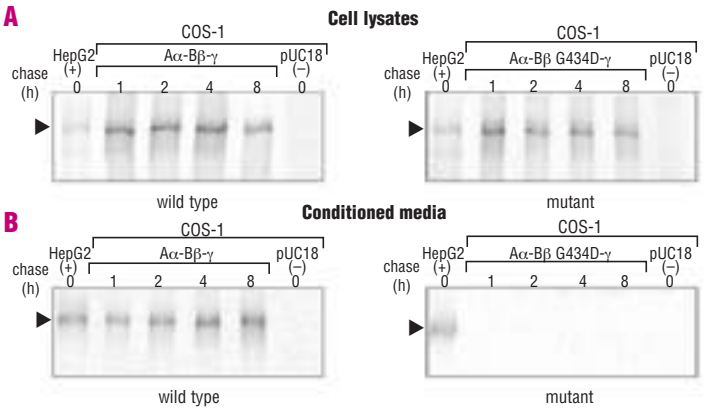


**Figure 2.** ***In vitro*** **expression of wild-type and G434D mutant fibrinogen. Immunoprecipitated fibrinogen from cell lysates (A) and conditioned media (B) of COS-1 cells transfected with equimolar mixtures of wild-type Aα and γ chains, together with either wild-type (left panels) or G434D Bβ (right panels) chains. Untransfected HepG2 cells and mock-transfected (pUC18) COS-1 cells represent the positive and negative controls, respectively. Cells were labeled with [$^{35}$S]-methionine and [$^{35}$S]-cysteine for 2 hours and subsequently chased for 0, 1, 2, 4, and 8 hours. Samples were separated on 4% SDS-PAGE under non-reducing conditions. The arrowhead indicates the 340-kDa hexameric fibrinogen molecule. Mutant fibrinogen is synthesized and assembled by the cells but fails to be secreted into the medium.**

## Discussion

In this study, we report a novel genetic defect causing severe hypofibrinogenemia in an Indian patient, born from a consanguineous marriage. The proband had severe bleeding manifestations (hemorrhage from the umbilical cord and menorrhagia) and she died recently as a consequence of a cerebral hemorrhage, an uncommon severe complication of type I fibrinogen deficiencies.[36] Sequence analysis of the three fibrinogen genes revealed a novel homozygous G to A transition in the Bβ-chain gene. The newly identified nucleotide variation results in a Gly→Asp missense mutation (G434D), whose pathogenic role is suggested by the extremely high degree of conservation of the involved residue among vertebrates (Figure 1B). The large and acidic aspartate residue introduced by this mutation substitutes a small, neutral glycine, most likely disrupting domain folding. Strikingly, Gly434 lies in the C-terminal globular D domain (residues 198-461) of the fibrinogen Bβ-chain (Figure 1A), a region where six of the seven Bβ-chain missense mutations so far reported as responsible for afibrinogenemia and hypofibrinogenemia are tightly clustered.[16,17,26-28,37] In fact, considering that the trimeric fibrinogen half-molecule measures approximately 230 Å in length, all but one of the missense mutations affecting the Bβ chain are localized in a region spanning only 20 Å. These data are consistent with the hypothesis that the D domain is essential for correct folding and secretion of fibrinogen, as recently confirmed by Vu and colleagues.[38]

In order to study the predicted pathogenic role of the G434D mutation, *in vitro* expression experiments were performed. Wild-type and G434D mutant fibrinogens were independently and transiently expressed in COS-1 cells, a cell line widely used to study fibrinogen assembly and secretion.[26,31] Pulse-chase experiments showed normal synthesis but altered secretion of the hexameric mutant molecule, which persisted intracellularly (Figure 2A,B). The observed complete impairment of fibrinogen secretion is somewhat in contrast with the antigen and functional fibrinogen levels measured in the proband's plasma. In fact, our experimental data are consistent with those described for afibrinogenemia causing missense mutations.[16,17,26] However, it should be stressed that the fibrinogen levels of the patient were assessed after a whole-blood transfusion and that her severe bleeding history is suggestive of afibrinogenemia rather than hypofibrinogenemia. In this regard it would have been particularly interesting to assess fibrinogen levels in the proband's consanguineous parents, who are expected to be heterozygous for the G434D mutation and, therefore, have *true* hypofibrinogenemia.

To further investigate the suggested role of G434D in impairing fibrinogen secretion, the sensitivity of wild-type and mutant fibrinogens to endoglycosidase H was assayed at different times after transfection. In fact, the N-linked oligosaccharides in the Bβ- and γ-fibrinogen chains normally become endoglycosidase-H resistant only when they are processed through the Golgi stacks and their oligosaccharide chains are mature.[5,39] Both mutant and wild-type Bβ and γ chains were sensitive to endoglycosidase H at each analyzed period (*data not shown*) suggesting that intracellular retention of the G434D fibrinogen is likely to result from a block during its post-translational pathway in the endoplasmic reticulum or, at the farthest, in the *cis*-Golgi, where endoglycosidase-H resistance is still not acquired.[35] The impossibility of distinguishing between the wild-type and the mutant protein is probably due to the quick post-endoplasmic reticulum processing that does not allow endoglycosidase-H resistant protein to be visible in the wild-type lysate while it is clearly recognizable in conditioned media. This approach is further complicated by the low efficiency of fibrinogen secretion by over-expressing COS-1 cells, resulting in retention of fibrinogen in the endoplasmic reticulum also in cells expressing the wild-type chains.[33]

Missense mutations affecting secretory proteins are known as possible causes of endoplasmic reticulum storage diseases (ERSDS), characterized by plasma deficiency and by hepatic storage of the corresponding protein.[40] Fibrinogen missense mutations are good candidates for causing hepatic storage disease, even though this phenotype has been associated with such muta-

tions in only two cases.[41,42] This is due to the fact that ERSDS require years to become clinically evident and that liver specimens from patients with coagulation defects are not commonly available for ethical reasons, thus hampering an unambiguous diagnosis of ERSD.[33] The results of the expression studies and endoglycosidase-H sensitivity assays described in this paper are compatible with an ERSD phenotype, but this outcome of G434D remains highly hypothetical and not verifiable *in vivo*. In conclusion, the elucidation of the pathogenic role of the G434D mutation increases the number of naturally occurring mutations that support the structural importance of the Bβ-chain D domain in fibrinogen assembly and secretion.

*All the authors participated in the conception and design of the present study, in the analysis and interpretation of data, and in revising the manuscript. LM was responsible for DNA sequencing, mutation detection, site-directed mutagenesis, cells transfection, interpretation of results, and drafting the manuscript. RA and SD were responsible for metabolic labeling, immunoprecipitation, SDS gel electrophoresis, Endo-H treatment, interpretation of results and correction of the manuscript. FP performed the coagulation testing and supervised the clinical characterization of the patient. KG enrolled the patient in the study, collected the clinical history and evaluated the clinical phenotype. MM was involved in the discussion of the results and in the correction of the manuscript.*

*The order in which the names of the authors appear is based on their contribution to the study. MLT, as supervisor and main person responsible for the entire work, is cited last. The authors reported no potential conflicts of interest. The financial support of Telethon – Italy (grant no. GGP030261) is gratefully acknowledged. This work was supported by FIRST (Fondo Interno Ricerca Scientifica e Tecnologica), and IRCCS Maggiore Hospital, Milan, Italy and partially funded by a grant from the Fondazione Italo Monzino to FP.*

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