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Letter to the Editors-in-Chief

Clinical and molecular characterization by next generation sequencing of Spanish patients affected by congenital deficiencies of fibrinogen

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Dear Editors-in-chief,

Congenital deficiencies of fibrinogen (CDFs) are coagulation disorders characterized by a reduced quantity and/or quality of circulating fibrinogen. CDFs can be classified as type I: complete absence or markedly reduced fibrinogen levels (afibrinogenemia or hypofibrinogenemia); or type II disorders: dysfunctional circulating protein, (dysfibrinogenemia or hypodysfibrinogenemia) [1]. Fibrinogen deficiency can be discovered at any age, but afibrinogenemia usually manifests early in childhood, often in the neonatal period [2]. The prevalence of afibrinogenemia is estimated at about 1 to 1 per million, whereas the prevalence for hypo and dysfibrinogenemia is difficult to establish because of a large number of asymptomatic cases.

Fibrinogen is encoded in *FGA*, *FGB*, and *FGG* genes, clustered in a region of 50 kb on chromosome 4q28–31.3 [3]. At present, approximately 200 different missense mutations within fibrinogen gene cluster have been described in the literature. Among them, 40% were hypofibrinogenemia, whereas 50% had disfibrinogenemia [4].

The aim of our study was to characterize Spanish patients with CDF through the complete *FGA*, *FGB* and *FGG* gene sequencing by Next generation sequencing (NGS), to contribute to a further understanding of the biology of the defects in this key coagulation factor.

1. Patients and methods

Routine coagulation tests, including the activated partial thromboplastin time, prothrombin time and thrombin time were performed in BCSXP automated coagulometer (Siemens Healthcare, Marburg, Germany). Functional fibrinogen was tested with the reagent Multifibren U (Siemens Healthcare) accordingly to the manufacturer instructions. The immunologic fibrinogen level was measured with ELISA using a matched-pair antibody set of human fibrinogen antigen (Enzyme Research Laboratories, South Bend, IN, USA). Fibrinolysis studies were performed on a spectrophotometer (Shimadzu, Kyoto, Japan). A polymerization/degradation curve was recorded. The ratio between fibrinogen formation and degradation velocities was then calculated. A high ratio may indicate resistance to fibrinogen lysis. Conversely, a decreased ratio may indicate an increased lysis.

Genomic DNA was isolated from peripheral blood extracting kits following standard procedure (QIAsymphony, Qiagen, Hilden, Germany). A custom SureSelect panel (Agilent Technologies, Santa Clara, CA, USA), was designed including the *FGA*, *FGB* and *FGG* gene, using SureDesign Custom Design Tool provided by Agilent Technologies. An adaptor-tagged DNA library was purified, amplified and enriched using SureSelect QXT capture library (Agilent Technologies, Santa Clara, CA, USA). Sequencing of the libraries was performed on a MiSeq instrument (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. The sequences were aligned and mapped against human genome version 19 by Sure Call (Agilent Technologies Santa Clara, CA, USA). Pathogenicity of unknown variants was predicted by in silico analysis using software and following the American College of Medical Genetics and Genomics (ACMG) criteria. All variants detected were confirmed by Sanger sequencing.

Seventeen unrelated patients were included. The patients' characteristics are summarized in Table 1. Written informed consent was obtained from all the participants according to the Ethics Committee of the Hospital Universitari i Politècnic La Fe (Valencia, Spain). All the patients had normal levels of other hemostatic parameters. Circulating and lupus anticoagulants were negative. There was no evidence of abnormalities in the liver and kidney.

2. Results

All patients showed one or more variants in *FGA*, *FGB or FGG* genes. Genetic findings are summarized in Table 2.

In the 17 patients analyzed, we found several genetic variants associated with CDFs: a great deletion in *FGA* gene affecting exons 2–6 that is consistent with a recurrent large 11 kb deletion previously described [5], a novel small deletion in *FGA* gene (c.1441delG in *FGA* gene), two splicing variants (a novel one; c.606G > A in a exonic region of *FGB* gene), a new stop codon variant (p.Tyr288Ter in *FGG* gene) and eleven missense variants (five novel ones; p.Lys71Thr, p.Arg114Lys, p.Trp334Cys, p.Asn335Tyr in *FGA* gene, and p.Tyr374Cys in *FGG* gene).

A total of 8 novel causative genetic variants were detected, most of them found in unique families. Bioinformatic prediction for thenovel

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Table 1
Phenotype of probands. Gender F is female and M male. Lysis ratio shows the ratio between polymerization and degradation of fibrinogen, normal range is 1.80 ± 0.42. NV means 'not valuable'.

Patient	Gender	Age (years)	Classification	Lysis Ratio	Clinical phenotype
P1	F	56	Hypofibrinogenemia	2.11	Asymptomatic
P2	M	32	Afibrinogenemia	NV	Haemorrhagic. Umbilical cord haemorrhage, muscular hematomas, epistaxis, hemarthros and surgery bleeding
P3	F	40	Dysfibrinogenemia	NV	Haemorrhagic. Bleeding during tooth extraction and postpartum bleeding
P4	F	39		NV	Haemorrhagic and thrombotic. Muscular hematomas, hematuria, epistaxis, gingivorrhages and hemarthros. Peripheral arterial thrombosis and foot vasculitis
P5	M	44	Afibrinogenemia	NV	Haemorrhagic. Cephalohematoma, easy bruising, gingivorrhage and bleeding during tooth extraction
P6	F	56	Afibrinogenemia	3.05	Asymptomatic. Parental history of thrombosis
P7	F	20	Dysfibrinogenemia	NV	Haemorrhagic. Easy bruising after traumatisms and moderate to heavy menstrual periods. Parental history of bleeding
P8	F	17	Dysfibrinogenemia	3.25	Haemorrhagic. Epistaxis and menorrhagia. Parental history of bleeding
P9	F	7	Dysfibrinogenemia	4.95	Asymptomatic
P10	F	10	Hypofibrinogenemia	1.32	Asymptomatic
P11	F	57	Dysfibrinogenemia	2.15	Haemorrhagic. Gingivorrhage. Parental history of bleeding
P12	F	19	Hypofibrinogenemia	7.67	Asymptomatic. Parental history of thrombosis
P13	F	16	Hypofibrinogenemia	7.12	Asymptomatic. Parental history of thrombosis
P14	M	46	Dysfibrinogenemia	1.58	Haemorrhagic/Asymptomatic. One bleeding after exodontia
P15	F	20	Hypofibrinogenemia	2.12	Asymptomatic
P16	F	72	Hypofibrinogenemia	0.53	Haemorrhagic. Easy bruising, heavy menorrhagia and surgery bleeding
P17	M	58	Dysfibrinogenemia	3.10	Asymptomatic

Table 2
Genotype of probands. FGA reference is NM_000508.3; FGB reference is NM_005141.4; FGG reference is NM_021870.2. Classification following ACMG criteria was obtained with the prediction software VarSome (http://varsome.com/) which relies on DANN, GERP, Mutation Taster, Mutation Assessor FATHMM, LRT, MetaLR, MetaSVM, and PROVEAN between other prediction software.

Patient	Gene	Nucleotide change	Protein change	Carrier Status	Described	Classification
P1	FGB	c.606G > A	p.Leu202Leu	Homozygous	No	VOUS
P2	FGA	delE2-E6		Homozygous	Neerman-Arbez M 1999	Pathogenic
P3	FGA	c.1441delG	p.Glu481LysfsTer3	Heterozygous	No	Likely pathogenic
	FGA	c.510 + 1G > T		Heterozygous	Neerman-Arbez M 2000	Pathogenic
P4	FGA	c.510 + 1G > T		Homozygous	Neerman-Arbez M 2000	Pathogenic
P5	FGA	delE2-E6		Homozygous	Neerman-Arbez M 1999	Pathogenic
P6	FGG	c.902G > A	p.Arg301His	Heterozygous	Kehl M 1984	Likely Pathogenic
P7	FGG	c.1007 T > C	p.Met336Thr	Homozygous	Yamazumi K 1989	VOUS
P8	FGG	c.901C > T	p.Arg301Cys	Homozygous	Terukina S 1987	Likely Pathogenic
P9	FGG	c.1201C > T	p.Arg401Trp	Heterozygous	Brennan SO 2002	Likely Pathogenic
P10	FGA	c.103C > T	p.Arg35Cys	Heterozygous	Soria J 1982	Likely Pathogenic
P11	FGG	c.864 T > G	p.Tyr288Ter	Heterozygous	No	Pathogenic
P12	FGG	c.1121A > G	p.Tyr374Cys	Heterozygous	No	Pathogenic
P13	FGA	c.341G > A	p.Arg114Lys	Heterozygous	No	VOUS
P14	FGG	c.323C > G	p.Ala108Gly	Heterozygous	Brennan SO 2000	VOUS[a]
P15	FGA	c.212A > C	p.Lys71Thr	Heterozygous	No	Likely Pathogenic
P16	FGA	c.1002G > T	p.Trp334Cys	Heterozygous[b]	No	Likely Pathogenic
	FGA	c.1003A > T	p.Asn335Tyr		No	Likely Benign
P17	FGG	c.902G > A	p.Arg301His	Heterozygous	Kehl M 1984	Likely Pathogenic

[a] Variant FGG p.Ala108Gly was predicted as VOUS, but in Clinvar is described as pathogenic.
[b] Both variants were found in cis.

missense variants has concluded that *FGG* p.Tyr374Cys, *FGA* p.Lys71Thr and *FGA* p.Trp334Cys have a deleterious effect. Meanwhile, *FGA* p.Asn335Tyr is predicted as likely benign, although it is a residue well conserved with a GERP score of 5.25.

3. Discussion

Monoallelic and biallelic mutations in *FGA*, *FGB*, and *FGG* genes are associated with different inherited disorders, reflecting the pleiotropic function of the fibrinogen protein [6]. Patients that are diagnosed with CDF condition are either asymptomatic, or can suffer bleeding symptoms, thrombophilia, or even both.

The frequency of CDFs in the general population is very low. In our study we have detected three cases of afibrinogenemia two of them caused by a known large deletion of *FGA* gene comprising from exon 2 to 6 (P2, P5), while the other patient was Homozygousozygous for a recurrent splicing variant, c.510 + 1G > T in *FGA* gene (P4). Both variants are transmitted in an autosomal recessive fashion and with a severe bleeding phenotype. Indeed, P2 was diagnosed at the time of delivery by heavy bleeding from the umbilical cord. Patient P4, despite having a significant hemorrhagic clinic, has also presented peripheral arterial thrombosis that requires anticoagulation and antiaggregation prophylaxis at the same time with fibrinogen concentrates. Afibrinogenemias, although rarely, can also present thrombotic symptoms, which emphasizes the clinical diversity of fibrinogen alterations. One possible explanation for this variability is the existence of modifier genes/alleles.

The most frequent variants found in our work are missense variants in exon 8 of *FGG* gene. Three of them resulted in dysfibrinogenemia (p.Arg301His; p.Arg301Cys; p.Met336Thr) and one was associated to hypofibrinogenemia (p.Tyr374Cys). The p.Arg301His variant is a frequent and known variant in congenital dysfibrinogenemia [7]. A novel

stop codon variant, p.Tyr288Ter, in exon 8 of *FGG* gene resulted in hypofibrinogenemia due to deficiency of protein, leading to a bleeding phenotype.

We have described five novel missense variants in *FGA* gene (p.Arg114Lys; p.Lys71Thr; p.Trp334Cys; p.Asn335Tyr) and in *FGG* gene (p.Tyr374Cys). *FGA* p.Lys71Thr and *FGG* p.Asn335Tyr resulted in hypofibrinogenemia and the rest of variants caused dysfibrinogenemia. A novel small deletion in *FGA* gene (c.1441delG) has been detected in a heterozygous compound status in a proband (P3) with bleeding phenotype and diagnosed as dysfibrinogenemia.

Knowing the genetic alteration, together with the functional studies of the fibrinogen and the familial and personal history of the patients, help us to establish the greater or lesser hemorrhagic or thrombotic tendency of our patients and to consider a better therapeutic approach. In this sense, NGS has proved to be a useful and reliable tool for sequencing simultaneously all the genes potentially involved in the disease.

Authorship

AM, AZ and MI drafted the manuscript. AM designed and performed the molecular studies. AZ and MI performed the molecular analyses. SB, JC and AZ supervised clinical and experimental findings. ARC, SH, AB and FF were involved in the management of the patients and provided the clinical data. SB and AZ were responsible for the conception of the study and final approval of the draft. All authors reviewed the draft and contributed to the final manuscript.

Declaration of Competing Interest

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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Andrés Moret[a], Ángel Zúñiga[b,*], Mariam Ibáñez[b,c,d], Ana Rosa Cid[a], Saturnino Haya[a], Fernando Ferrando[a], Amando Blanquer[a], José Cervera[b], Santiago Bonanad[a]

[a] *Unidad de Hemostasia y Trombosis, Servicio de Hematología, Hospital Universitario y Politécnico La Fe, Valencia, Spain*

[b] *Unidad de Genética, Servicio de Hematología, Hospital Universitario y Politécnico La Fe, Valencia, Spain*

[c] *Centro de Investigacion Biomédica en Red de Cáncer (CIBERONC), Instituto Carlos III, Madrid, Spain*

[d] *Departamento de Ciencias Biomédicas, Facultad de Ciencias de la Salud, Universidad CEU Cardenal Herrera, Valencia, Spain*

E-mail address: zunyiga_ang@gva.es (Á. Zúñiga).

* Corresponding author at: Unidad de Genética, Hospital Universitario y Politécnico La Fe, Avenida de Fernando Abril Martorell, 106, 46026, Valencia, Spain