Molecular characterization of 16 hemophilia B families in Aragon, Spain
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Molecular characterization of hemophilia B at gene level has become an indispensable tool for a proper genetic counseling in carriers and for a closer surveillance of inhibitor development in several severe forms. Our study was aimed at characterizing the molecular defects in the factor IX (FIX) gene in hemophilia B families in Aragon, a center-east region of Spain. Direct sequencing of all regions of likely functional significance of the FIX gene was performed in the screened 18 hemophilia B families. Quantitative techniques, such as multiplex ligation-dependent probe amplification reaction, were carried out only in patients in whom no mutation was found. We have identified the molecular events responsible for hemophilia B in 16 unrelated families (eight with mild hemophilia B and eight with severe hemophilia B). Out of all families studied, we have found 14 missense mutations and two nonsense mutations; still we were unsuccessful in determining the genetic defects in two severe and unrelated families. Of the 16 characterized mutations, 14 of them lie in the protease domain in which one mutation, A233T, was surprisingly found in three unrelated families. We also report and discuss the pathogenicity of F314L, a novel mutation found in the protease domain. Our molecular data reflect a notable heterogeneity of the mutational spectrum mainly in the protease domain of FIX. This is the first mutation report on the disease in Aragon, Spain. Blood Coagul Fibrinolysis 24:000–000 © 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Introduction
Hemophilia B (MIM# 306900) is an X-linked recessive coagulation disorder characterized by a reduction in the activity of the coagulation factor IX (FIX) that occurs roughly in one out of 30 000 live male births in all populations [1]. Depending on the residual activity of FIX, the disease is classified as mild (>5% and <30%), moderate (>1% and <5%), or severe (<1%) [2]. Patients with severe phenotype used to die not long after birth due to recurrent bleeding. Nowadays, due to new therapeutic achievements, for example, the recombinant FIX [3], even severe hemophilia B patients have a life expectancy similar to the healthy population [1,3,4].

Hemophilia B is mainly a consequence of genetic alterations produced in the FIX gene (F9, GenBank accession number: K02402.1). The FIX gene is 34 kb long and is located at Xq27.1 spanning 8 exons and 7 introns. All kind of genetic defects have been described, but point mutations are the most prevalent. More than 1000 point mutations have been described throughout the entire FIX gene and all of them have been thoroughly recorded on an internet mutation database [5]. The majority of them consist of missense mutations that affect the whole coding region [5]. The severity of the disease ensuing from a certain missense mutation depends on the type of the mutating residue (and its position in the protein sequence) and on the amino acid resulting from that change. Thus, two different missense mutations affecting the same residue may translate into different phenotypes and might even translate into an absence of any disease whatsoever [6]. In contrast, a nonsense mutation will always result in a severe phenotype due to a truncated protein translation [6].

FIX participates in the intrinsic coagulation pathway and its main role is factor X (FX) activation. The efficiency of this activation depends on the presence of factor VIIIa (FVIIIa), considered the natural cofactor of FIX [7]. FIX is translated in the endoplasmic reticulum into a poly-peptide that rapidly suffers diverse posttranslational modifications: \(\gamma\)-carboxylation, hydroxylation, sulfation, glycosylation, phosphorylation, and signal peptide and propeptide cleavage, which have been extensively described before [8–13]. Mature FIX is secreted as a 415 amino acid chain requiring activation by either factor VII/tissue factor (FVII/TF) complex or by factor XI [14,15]. FIX activation leads to a small fragment liberation, called the activation peptide, which transforms FIX into its active conformation (FIXa). FIXa is a heterodimer in which the two chains are covalently linked by a disulfide bond. Mature FIX has four differentiated
domains: γ-carboxyglutamic acid (Gla) domain, two epidermal growth factor (EGF)-like domains (EGF-1 and EGF-2), and the catalytic or protease domain.

In the present article, we have assessed the molecular events occurring in 18 hemophilia B families in Aragon, a middle-east region of Spain. The molecular defects responsible for hemophilia B have been determined in 16 families and could not be found in two families. Out of the 16 characterized mutations, 14 were missense mutations with A233T being elicited in three unrelated families. A novel mutation, F314L, has been discovered. However, and according to the FIX alignments from different species, F314L mutation’s role as disease causative agent is unclear as some species bear this amino acid variation in its FIX native sequence.

Methods
Hemophilia B patients from all parts of Aragon were recruited from the University Hospital Miguel Servet and gave their corresponding informed consent. FIX coagulation activity in every patient was determined by one-stage clotting assay. Eighteen patients from different unrelated families were included in the study. Ten of them were diagnosed as severe hemophilia B, whereas the other eight were classified as mild hemophilia B.

Typically, 5 ml of blood in EDTA tubes was collected per patient. The genomic DNA from the leukocytes was extracted with the QiAamp DNA Blood Mini Kit (Qiagen, Venlo, The Netherlands) following the manufacturer’s instructions. The DNA from the spin columns was eluted in nuclease free water (Invitrogen, Carlsbad, California, USA). This DNA was checked in a 1% agarose gel electrophoresis with Tris-Borate EDTA (TBE) buffer at pH 8.0. Only when the resulting amplicons were the expected sizes, were they subjected to the sequencing reaction.

The amplified fragments were then incubated with Exo-sap IT (GE Healthcare, Little Chalfont, UK) in order to clean the PCR reaction from the exceeding primers and nucleotides. The corresponding sequencing reactions were performed with the BigDye Terminator kit (Applied Biosystems, Foster City, California, USA) using the forward or the reverse primer. Different length fragments were purified from the remaining terminators by the DyEx 2.0 Kit (Applied Biosystems). The samples were then loaded into a 3130xl sequencer (Applied Biosystems) and after the run, the results were analyzed using Bioedit and GenomeLab GX (Beckman Coulter, Brea, California, USA) softwares.

When a presumed mutation was found in a specific region or exon, another amplification reaction of the corresponding region or exon was performed again. Following that, a new confirmatory sequencing reaction was performed with the primer not used in the first amplification.

The multiplex ligation-dependent prove amplification (MLPA) reaction was performed with the SALSA F9 Kit (MRC-Holland, Amsterdam, The Netherlands) as an external service and only in those patients in whom a mutation was not found.

The different protein sequences were obtained from the National Center for Biotechnology Information (NCBI) database and the protein sequence alignments were performed using Clustal Omega [17]. The crystallographic structures of FIX were downloaded from the Protein Data Bank and the images were created with Pymol software.

Results
Eighteen unrelated hemophilia B families from Aragon were studied: eight families with mild hemophilia B phenotype and ten with severe hemophilia B phenotype. All families were clinically classified as hemophilia B before the start of the study. However, the corresponding mutational events responsible for hemophilia B were unknown at that precise moment.

Direct sequencing for all the affected members was performed as a first step. When a mutation was found, an independent PCR was performed again. Following that, and in order to validate the mutation, a new sequencing reaction was performed with the other primer, that is, the one not used in the preceding reaction. Only in those cases in which no mutation was found, the gene of FIX was quantitatively analyzed by the MLPA technique.

Table 1 summarizes the molecular characterization of the 18 unrelated hemophilia B families screened along with some clinical data. The mutational events responsible for hemophilia B were found in 16 families, with 14 and two being missense and nonsense mutations, respectively. Fourteen of the characterized mutations affect the protease domain, one the propeptide and the other one the EGF-1 region (Fig. 1). A recurrent mutation, A233T, was detected in three unrelated families and a novel mutation, F314L was also found. Unexpectedly enough, no mutations were found in two severe hemophilia B families.

Discussion
Following thorough genetic analysis (direct DNA sequencing), we found the mutations responsible for hemophilia B, in 16 of the 18 hemophilia B families...
screened. Uneventful genetic analysis in two severe hemophilia B and unrelated families, even by performing the MLPA reaction, was completely unexpected due to the low prevalence of genetically undetermined cases in hemophilia B (around 1% of all individuals) [18]. These data reflect that there are uncovered determinants affecting FIX biogenesis at some point.

According to the FIX mutation database, point mutations in hemophilia B, that is, nonsense and missense mutations, are responsible for the 74% of the total characterized cases [5]. In the present study, we found that 100% of mutations found were indeed point mutations from which the 88 and 12% resulted in missense and nonsense mutations, respectively (Table 1). This percentage is not far from the 74% reported, but probably the most surprising data in the present study was the finding that the 88% of our mutations lay within the catalytic domain in contrast to the 46% reported in the FIX mutation database [5]. The distribution of the gene

Table 1 Molecular characterization of the studied 18 unrelated hemophilia B families in Aragon, Spain

<table>
<thead>
<tr>
<th>Mutation ID number</th>
<th>Nucleotide change</th>
<th>Exon</th>
<th>Amino acid change</th>
<th>Domain</th>
<th>Severity of disease</th>
<th>Referred (number of families)</th>
<th>Acquired inhibitors</th>
<th>Conservation level</th>
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<tr>
<td>HB1</td>
<td>6374g&gt;a</td>
<td>2</td>
<td>R-1K</td>
<td>Propeptide</td>
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<tr>
<td>HB2</td>
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<td>4</td>
<td>G60S</td>
<td>EGF-1</td>
<td>Mild</td>
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<td>Complete</td>
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<tr>
<td>HB3</td>
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<td>7</td>
<td>A233T</td>
<td>Protease</td>
<td>Mild</td>
<td>3</td>
<td>No</td>
<td>Partial</td>
</tr>
<tr>
<td>HB4</td>
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<td>8</td>
<td>R252X</td>
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<td>Complete</td>
</tr>
<tr>
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<td>8</td>
<td>T296M</td>
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<td>Complete</td>
</tr>
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<td>G305V</td>
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<td>Partial</td>
</tr>
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<td>8</td>
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<td>–</td>
<td>–</td>
<td>Severe</td>
<td>2</td>
<td>No</td>
<td>Complete</td>
</tr>
</tbody>
</table>

Fig. 1

(a) Factor IX domain architecture depiction. (a) Schematic representation of the FIX precursor from the N-terminal to C-terminal region. Both different domains and also the numeration of the first residue belonging to each domain are visible. The different mutations found in the present study are shown. (b) Crystal structure of porcine FIXa (PDB code: 1PFX). Note that the colored domains in (a) correspond to those in (b).
mutations detected in our study is clearly illustrated in Fig. 1.

We performed sequence alignments of FIX from different species (Fig. 2). Actually, we included a couple of primates very close to *Homo sapiens* in the phylogenetic tree but also other species that are more distant. These species were the chimpanzee (*Pan troglodytes*), the rhesus macaque (*Macaca mulatta*), the house mouse (*Mus musculus*), the chicken (*Gallus gallus*), and the zebrafish (*Danio rerio*), which represents the most phylogenetically diverged animal. A high degree of conservation of a certain residue within an enzyme is considered as an indicator of the importance of that residue from a structural point of view either in the enzymatic mechanism (as an integrating part of the catalytic triad) and stability or in a protein–protein interaction (as a part of its interface) [6]. The amino acids integrating some of the mutations found in the present study are completely conserved as in Arg-1, Gly60, Thr296, Cys336, Phe349, Gly367, Ser384, Gly386, and Glu387, whereas others are somewhat conserved as in Ala233 and Gly305; Phe314 is the least conserved of the reported mutations (Fig. 2 and Table 1).

**R-1K**

The R-1K mutation is a consequence of a g>a transition at the nucleotide 6374 in exon 2 and has been reported four times before [5]. The mutation lies in the propeptide domain, which is cut prior to the secretion of the mature FIX. The propeptide interacts with the γ-carboxylase, the enzyme responsible for the carboxylation of the glutamic acids in the Gla-domain. Arg-1 is the last amino acid of the propeptide domain and is completely conserved (not shown). Mutation of this arginine residue to other amino acids like threonine or serine leads to a severe phenotype [5]. In the native FIX sequence, the last four amino acids of the propeptide are Arg-Pro-Lys-Arg, three of them being positively charged amino acids at neutral pH. It seems that a conservative substitution of this Arg-1 into a lysine can partially overcome this issue.

![Fig. 2](image-url)
the resulting alteration, leading to the secretion of some amounts of normal mature FIX, explaining the patient’s mild hemophilia B phenotype. This is in good agreement with previously performed experiments in Chinese hamster ovary (CHO) cells, in which it was shown that R-1K mutation was partially processed during FIX biosynthesis [19].

**G60S**

This mutation is produced as a $g>a$ transition at the nucleotide 10430 in exon 4. The G60S mutation lies within the EGF-1 domain in which EGF is a common structural motif found in various extracellular proteins. EGF-like motifs are around 40 amino acid long, have 3 disulfide bonds and a calcium binding pocket at the N-terminal position. The Gly60 is completely conserved at protein level (not shown) and consistently with structural data available (PDB code: 1EDM) it is not involved in calcium binding [20]. According to the previously performed refolding experiments, the G60S mutant is able to reach the native calcium-bound state at least in some small quantities [21]. The EGF-1 interacts with the FVII/TF complex while it seems not to interact with FVIIIa. According to the FIX mutation database [5], this mutation is very common (it has been reported in more than 70 patients) and translates into a mild phenotype [5].

**A233T**

In the present study, a $g>a$ transition was observed at nucleotide position 30150 in three unrelated hemophilia B families. This point mutation is located in exon 7 and leads to a missense mutation in the protease domain, A233T, in which a hydrophobic amino acid like alanine is substituted by a polar one like threonine. In the FIX mutation database, this mutation is recorded as a frequent point mutation localized at nucleotide 31061 in exon 8 and is a conservative mutation at protein level as it can be seen in Fig. 2.

**T296M**

The T296M mutation is a consequence of a c$>t$ transition at nucleotide 31008 in exon 8. The mutation lies in the center of an $\alpha$-helix that directly interacts with FVIIIa and is almost completely buried [23,24]. In this mutation, a polar amino acid like Thr296 is replaced by an hydrophobic amino acid like methionine. As a result, one hydrogen bond is abolished [22]. According to the FIX mutation database, this mutation has been described in more than 100 individuals and is classified as producing a mild phenotype with varying antigenic levels [5]. This residue is completely conserved at protein level as it can be seen in Fig. 2.

**G305V**

A $g>c$ transversion at nucleotide 31055 in exon 8 was found in one hemophilia B family. This mutation has been reported only once in the past and its phenotype is also described as severe [5,18], consistent with our patient’s diagnosis. In this mutation, a glycine is substituted by a valine. Glycine is a small amino acid and the only amino acid with no real side chain, whereas valine is larger and hydrophobic. The severity of the disease could be related to a conformational destabilization of FIX as Gly305 is completely buried within the protease domain and is replaced by a bigger amino acid. This glycine is only partially conserved at protein level (Fig. 2).

**F314L**

The only novel mutation found in the present study is localized at nucleotide 31061 in exon 8 and is a consequence of a t$>c$ transition that produces the F314L mutation in the protease domain. According to the FIX mutation database, this mutation has not been registered or described yet [5]. However, a F314I mutation has been described in one patient with a moderate phenotype and a F314S mutation in three patients with mild phenotype [5].

**R252X**

The nonsense R252X was identified as a transition of a c$>t$ at the nucleotide 30875 in exon 8. Due to this mutation, the codon changes from CGA, codifying arginine, into the stop codon TGA. As a nonsense mutation, it would be expected that a truncated glycoprotein was formed leading to a severe phenotype as recorded in the FIX mutation database [5]. The mutation has been found in more than 50 affected patients. In spite of being a nonsense mutation, it has not been linked to inhibitor development up to present (Table 1).
In this patient, no other mutational event was found. If we only take into account the mutational data available in the FIX mutation database [5], the F314L mutation has to be considered as a mutation producing hemophilia B mild phenotype. Nevertheless, if we do consider the sequence alignment analysis (Fig. 2), the F314L mutation might not be responsible for hemophilia B. In this scenario, the real mutational event producing the hemophilia B mild phenotype remains unresolved and would have to be uncovered.

**C336Y**

The C336Y mutation is produced due to a transition of a g>a at the nucleotide 31128 in exon 8. According to the FIX mutation database, it has been described in less than 10 patients [5] and we have found in one studied family. This mutation lies in an α-helix of the protease domain, which is involved in the interaction with FVIIIa. All the cysteine residues in FIX are completely conserved across the species (Fig. 2). But the most important issue about this mutation is that it disrupts a disulfide bond in the protease domain. The mutation would limit FVIIIa interaction as a huge perturbation is produced in the region close to the mutation site. In addition, the presence of an odd number of cysteines would probably lead to the accumulation of nonnative disulfide bonds in the folding pathway of FIX due to the presence of a single cysteine [25]. This nonnative FIX forms may be prone to degradation by the intracellular machinery leading to a nonsecreted FIX, hence with undetectable FIX antigen [5].

**R338X**

A transition of c>t was found in one family at the nucleotide 31133 in exon 8. Again, as in the R252X mutation, the codon is changed from CGA that codifies arginine into the stop codon TGA. It is worth stressing that this mutation is not described in the FIX mutation database but in a scientific paper published in 2003 [26]. As a nonsense mutation, the resulting phenotype is classified as severe due to the formation of a truncated glycoprotein. As in the case of R252X, the patient has not developed any inhibitors (Table 1).

**F349L**

The F349L is a consequence of a t>c transition at the nucleotide 31166 in exon 8. Both of them are hydrophobic amino acids, but phenylalanine is aromatic, whereas leucine is aliphatic and smaller compared to phenylalanine. According to the FIX mutation database, this mutation has been described in three patients with a severe phenotype [5]. The Phe349 is completely buried in the protease domain and close to the two α-helices that interact with FVIIIa. In addition, this residue is completely conserved at protein level reflecting its high importance (Fig. 2). Perhaps, mutation of this amino acid would reorient the two adjacent α-helices and reduce its interaction with FVIIIa.

**G367A**

An g>c transversion at the nucleotide 31221 in exon 8 was found in one of the screened hemophilia B families. This point mutation leads to a G367A mutation in the protease domain. The Gly367 is located in the core of the protease domain, is only two residues from the catalytic Ser365, and is completely conserved (Fig. 2). In the FIX mutation database, this mutation has been reported in a single case and linked to a moderate hemophilia B phenotype [5] with an antigenic level of around 100%. Our patient has been diagnosed as having mild hemophilia B phenotype. Perhaps, the physiopathology of this mutation may be a consequence of a conformational destabilization that is produced in the protease domain. This G367A mutation is somewhat a conserved one and it would appear that normal quantities of FIX are produced and secreted supporting the conformational destabilization hypothesis.

**S384R**

A g>c transversion at the nucleotide 31273 in exon 8 was found in one patient. This mutation leads to a missense mutation at protein level, S384R, in which the Ser384 is less than 3Å from the Asp269 of the catalytic triad. Moreover, the Ser384 is completely conserved across species (Fig. 2) and according to the FIX mutation database, this mutation has been described only once.
The severe phenotype that this mutation produces in our patient is probably consequence of its proximity to the catalytic triad and the perturbation produced on it as a relatively small and polar amino acid, like Ser384, is mutated to a long and positively charged amino acid like arginine.

**G386S**

We have found a g>a transition at nucleotide 31277 in exon 8 that produces a G386S missense mutation in the protease domain. Gly386 is also completely conserved at protein level (Fig. 2) and has been previously identified in two severe hemophilia B patients [5]. There is no data available concerning the antigenic FIX levels. However, other mutations have been reported in this residue (G386D, G386V, and G386A) and they have been linked to moderate and severe hemophilia B phenotypes [5]. In the case of G386D, the registered antigenic FIX levels are normal [5].

Gly386 is located at the entrance of the S1 pocket, the cleft at which FX is bound to FIX in the protease domain. The substitution of this glycine by a bigger and polar amino acid-like serine would drastically disturb the S1 pocket giving rise to the severe hemophilia B phenotype. Probably, this change would impair FX binding without affecting the secretion of FIX as in G386D mutant, in which high levels of antigenic FIX can be found.

**E387A**

The a>c transversion at nucleotide 31281 in exon 8 was found in one of the screened patient. This nucleotide change produces the E387A missense mutation, which has been described in six patients [5]. Glu387 is completely conserved at protein level (Fig. 2) and this mutation in our patient leads to a severe hemophilia B phenotype. In this case, a glutamic acid, which has a long and a negatively charged side chain at neutral pH is mutated to an alanine, one of the smallest amino acids, as it only bears an hydrophobic methyl group. According to the crystal structure (PDB: 1RFN), Glu387 interacts with Thr340 through a hydrogen bond and with Lys394 through a salt bridge. As alanine is hydrophobic, those interactions are supposed to be completely destroyed. Thr340 is close to one of the α-helices involved in FVIIa binding and there is strong evidence indicating that Lys394 coordinates Na$^+$ in a proposed sodium site [27], which seems important in catalysis and also in FVIIa binding [27]. Hence, the pathophysiology of this mutation could be related to a decreased FVIIa binding, while the antigenic FIX levels are normal [5].

**Conclusion**

In the present study, we have characterized the molecular defects in 16 unrelated Spanish hemophilia B patients. It is noticeable that the genetic alterations of two unrelated and affected families, with severe hemophilia B, have not been found even after performing quantitative techniques. All the mutations (100%) are a consequence of point mutations leading to missense mutations (88%) and nonsense mutations (12%). Remarkably, almost all the mutations lie within the catalytic domain (88%), in which a recurrent mutation, A233T, has been found in three unrelated patients.

Finally, we have found a novel mutation in the protease domain: F314L, which is quite striking. The Phe314 residue is not only nonconserved across species, as it can be proved from the performed sequence alignments (Fig. 2), but appears with this F314L variation in the wild-type FIX of *Gallus gallus*. This evidence raises the question of whether this mutation is really responsible for the patient’s mild phenotype (although no other mutational event was found in the patient). Therefore, we have started investigating this mutation by Molecular Dynamic simulations in the hope that it may shed some light on the real pathogenicity of F314L.

**Acknowledgements**

X.A-M. performed and designed the research, analyzed the data, and wrote the article. S.S.C. and R.R. performed the molecular biology techniques alongside X.A-M. J.F.L. designed the research and C.A. wrote some parts of the article.

**Conflicts of interest**

The authors state that they have no interests that might be perceived as posing a conflict or bias.

**References**