Genotype-phenotype correlation in combined deficiency of factor V and factor VIII

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Combined deficiency of factor V and factor VIII (F5F8D) is caused by mutations in one of 2 genes, either *LMAN1* or *MCFD2*. Here we report the identification of mutations for 11 additional F5F8D families, including 4 novel mutations, 2 in *MCFD2* and 2 in *LMAN1*. We show that a novel MCFD2 missense mutation identified here (D81Y) and 2 previously reported mutations (D89A and D122V) abolish MCFD2 binding to LMAN1. Measurement of platelet factor V (FV) levels in 7 F5F8D patients (4 with *LMAN1* and 3 with *MCFD2* mutations) demonstrated similar reductions to those observed for plasma FV. Combining the current data together with all previous published reports, we performed a genotype-phenotype analysis comparing patients with *MCFD2* mutations with those with *LMAN1* mutations. A previously unappreciated difference is observed between these 2 classes of patients in the distribution of plasma levels for FV and factor VIII (FVIII). Although there is considerable overlap, the mean levels of plasma FV and FVIII in patients with *MCFD2* mutations are significantly lower than the corresponding levels in patients with *LMAN1* mutations. No differences in distribution of factor levels are observed by sex. These data suggest that MCFD2 may play a primary role in the export of FV and FVIII from the ER, with the impact of LMAN1 mediated indirectly through its interaction with MCFD2. (Blood. 2008;111:5592-5600)

Introduction

Combined deficiency of factor V and factor VIII (F5F8D) is an autosomal recessive disorder characterized by simultaneous reduction in the levels of factor V (FV) and factor VIII (FVIII) activity and antigen and mild-to-moderate bleeding symptoms.[1] The reported levels of FV and FVIII in F5F8D patients range from as low as 1% to as high as 46% of normal, but generally fall between 5% and 30%.[1] Positional cloning identified 2 genes that are associated with the disorder.[2,3] Mutations in *LMAN1* account for approximately 70% of F5F8D families, while mutations in *MCFD2* account for the remaining 30%.[4-6] LMAN1 is a type-1 transmembrane protein that cycles between the endoplasmic reticulum (ER) and the ER-Golgi intermediate compartment (ERGIC).[7,8] It contains a mannose-specific carbohydrate recognition domain on the ER lumenal side and ER exit and retrieval motifs on the cytoplasmic side.[9] MCFD2 is an EF-hand domain protein that interacts with LMAN1 in a Ca^{2+}-dependent manner.[3] The LMAN1-MCFD2 protein complex functions as a cargo receptor that facilitates the transport of FV and FVIII from the ER to the Golgi apparatus.[3,10]

All the LMAN1 mutations reported to date are null mutations with the exception of a cysteine-to-arginine mutation that disrupts a disulfide bond that is required for its oligomerization and also destabilizes the protein.[6] In contrast, both null mutations and missense mutations have been identified in MCFD2. All 4 MCFD2 missense mutations reported to date change highly conserved amino acid residues in the EF hand domains,[3,6,11] and 2 have been shown to abolish LMAN1 binding,[3] indicating that LMAN1 and MCFD2 must function as a unit to transport FV and FVIII. Although MCFD2 appears to directly interact with FVIII,[10] it is unclear whether LMAN1 binds directly to FV/FVIII or indirectly via MCFD2.

Here we report identification of mutations in *MCFD2* and *LMAN1* in an additional 11 F5F8D families. With the availability of these and other published data on a large number of patients with known mutations and the corresponding FV/FVIII levels, we also address the question of whether there is a phenotypic difference between patients with *LMAN1* mutations and those with *MCFD2* mutations, and whether FV in plasma and FV in platelets are similarly affected.

Methods

Patients and mutation analysis

Table 1 lists the 15 affected individuals studied from 11 new families with affected individuals. Peripheral blood samples were obtained with written informed consent from probands and family members after diagnosis of F5F8D in accordance with the Declaration of Helsinki. FV and FVIII activities were measured at local clinical laboratories using one-stage

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assays based on prothrombin time (for FV) and activated partial thromboplastin time (for FVIII). For families B19 to B22 and B26 to B28, amplification by polymerase chain reaction (PCR) of exons and intron-exon junctions of the *LMAN1* and *MCFD2* genes and DNA sequencing were performed as reported previously.[2,3] For families B23 to B25, the presence of previously described mutations in Tunisian and Middle Eastern Jewish populations was confirmed by PCR amplification and restriction analysis as previously reported.[12]

Construction of MCFD2 expression vectors

Cloning of wild-type MCFD2 and MCFD2^{D129E} into the pcDNA3.1-myc-his expression vector was described previously.[3] The Stratagene mutagenesis II kit (La Jolla, CA) was used to introduce individual point mutations into the wild-type MCFD2 expression vector. The mutagenesis primers were as follows (only the sense primers are listed): D81Y, CAGCTCCATTACTTCAAAATGCATTATTATGATGGCAATAATTTGCTTG; D89A, GATGGCAATAATTTGCTTGATGGCTTAGAACTCTCCACA; and D122V, AAGATGAACTGATTAACATAATAGTTGGTGTTTTGAGAGATGATGAC. The presence of the desired mutation and the absence of a second mutation were verified by DNA sequencing.

Metabolic labeling and immunoprecipitation

COS-1 cells were transfected with the wild-type and different mutant MCFD2 expression vectors and labeled with ^{35}S methionine-cysteine (TRAN^{35}S LABEL; MP Biomedicals, Solon, OH) at 28 hours after transfection as previously described.[3] Immunoprecipitation with antimyc, anti-MCFD2, and anti-LMAN1 antibodies was performed as previously described.[10] Proteins were separated in 4% to 12% Criterion Bis-tris gels run with the Mes running buffer (Bio-Rad, Hercules, CA) and visualized by exposing to a Kodak Bio-Max film (Rochester, NY) using a Kodak Biomax Transcreen LE.

Immunofluorescence staining

Immunofluorescence staining of HeLa cells transfected with different MCFD2 expression vectors was previously described.[3] Images were viewed on a Leica DMRXE confocal microscope (Wetzlar, Germany) using a 40× oil-immersion objective with a 1.25 numeric aperture. Confocal images were acquired using the Leica Confocal Software, version 2.61.

Genotyping of the O blood type

The ABO glycosyltransferase gene around the common O1 allele (c.261delG) was amplified using primers MO-46 and MO-57 as described.[13] The region around the rare O2 allele (c.G802A) was amplified using the following primers: O2-S, AGATCCTGACTCCGCTGTTC; O2-AS, CACAAGTACTCGGGGGAGAG. The presence of a homozygous O allele, as detected by DNA sequencing, was scored as blood type O. Other genotypes were not distinguished further and scored as non-O. In our patient samples, no O2 genotype was observed.

Isolation of platelets and platelet FV assay

Washed human platelets were obtained from 7 individuals from 5 Italian families (Table 4), with mutations in *LMAN1* or *MCFD2,* and from 15 healthy subjects, as previously described.[14] Briefly, blood was collected in ACD (85 mM trisodium citrate, 71 mM citric acid, and 111 mM dextrose, pH 4.5) and centrifuged at 400*g* for 15 minutes at 20°C to obtain platelet-rich-plasma (PRP). After removal of platelet-poor plasma, platelet pellets were washed 3 times with Tyrode-17.5% albumin solution with 1 μM PGE1 as an inhibitor of platelet activation and then resuspended at 10^9/mL in the same buffer supplemented with 1 μM PGE1 and 1 μL/mL apyrase (kindly provided by Dr R. L. Kinlough-Rathbone, McMaster University, Hamilton, ON) and 1 mM PMSF (phenylmethanesulphonyl fluoride). FV antigen levels were determined in both plasma and platelet lysates by an enzyme-linked immunosorbent assay (ELISA) using a sheep anti-FV antibody (Affinity Biologicals, Ancaster, ON). Plasma FV and platelet FV were normalized to the hematocrit and platelet counts, respectively, to compare the relative reduction of FV in each pool among F5F8D patients and controls. Of note, the size of the platelet (plt) FV pool in our study (~ 3.7% of total circulating FV, or ~ 637 ng/10^9 plts in healthy controls) is significantly smaller than in previous reports (2500-7730 ng/10^9 plts by a radioimmunoassay[15] and 900-1300 ng/10^9 plts by a polyclonal antisera-based ELISA[16]). This discrepancy could be due to intrinsic differences in antigenicity between platelet and plasma FV. Consistent with this notion, FV has been shown to be substantially modified in platelets.[17,18]

Statistical analysis

We compiled a list of the current and all previously reported patients with known mutations in either *LMAN1* or *MCFD2* and their corresponding FV and FVIII levels (Tables 2,3). In cases where FV and FVIII levels were measured multiple times, only the means are listed. Two-tailed Student *t* test for independent samples, assuming equal variance, was used to determine differences between the mean activity levels. The relationship between FV and FVIII was measured using the Pearson correlation test.

Results

Mutation analysis in additional F5F8D patients

We analyzed 15 patients with F5F8D from 11 previously unreported families. Table 1 summarizes the factor V and factor VIII levels of the affected individuals. The proband in family B19 is of Afro-Caribbean origin. Family B20 is a consanguineous Turkish family with 2 affected siblings. Family B21 is an Iraqi Chaldean family. Family B22 is from Saudi Arabia. Families B23 to B25 are 3 unrelated Iranian Jewish families. Families B26 to B28 are from Italy. A novel homozygous *MCFD2* mutation was identified in family B19 (c.374-375insGA). This mutation occurs in exon 3 and leads to frameshift and a premature stop of translation. A novel homozygous *MCFD2* missense mutation (c.241G>T) was identified in family B22. This mutation causes a substitution of tyrosine for a highly conserved aspartic acid residue at amino acid position 81 (D81Y). Novel homozygous *LMAN1* mutations (c.795delC and c.1356delC) were identified in both affected siblings in families B20 and B21, respectively. A previously reported mutation (c.149+5G>A) was identified in both affected siblings of family B27. The *LMAN1* mutations in families B20 and B21 are single-nucleotide deletions in exon 8 and exon 11 that both result in a frameshift and a premature stop of translation. Analysis of families B23 to B25 identified homozygosity in all affected individuals for 2 previously reported mutations (c.1149+2T>G and c.89–90insG).[2] The proband of family B26 carries a mutation (c.2T>C) commonly found in patients of Italian origin.[19] Although no mutations were identified by DNA sequencing of the *MCFD2* and *LMAN1* genes in family B28, Western blot analysis demonstrated the absence of detectable LMAN1 protein (data not shown), suggesting an *LMAN1* regulatory or splice mutation missed by the exonic sequence analysis.

All known missense mutations in MCFD2 abolish LMAN1 binding

The D81Y mutation is one of 5 missense mutations identified in MCFD2 to date, all of which result in an amino acid substitution at a highly conserved amino acid residue in one of the 2 EF hand domains. Two of these missense mutations (D129E and I136T), both located in the second EF hand domain, have been shown to disrupt LMAN1 binding.[3] To test the effect of the remaining 3 missense mutations (D81Y and 2 previously reported mutations, D89A[6] and D122V[11]), each was expressed as a myc-tagged fusion protein and transfected into COS-1 cells. Antimyc antibody was

Table 1. *LMAN1* and *MCFD2* mutation analyses, geographic origins, sex, and FV/FVIII levels in new F5F8D patients included in the current study

Patient	Origin	Sex	FV level	FVIII level	LMAN 1 mutation	MCFD2 mutation
B19	Afro-Caribbean	F	4	9	N/D	c.374-375insGA
B20-1 (c)	Turkey	M	35	15	c.795delC	None
B20-2 (c)	Turkey	M	25	20	c.795delC	None
B21-1	Iraq	F	12	18	c.1356delC	None
B21-2	Iraq	M	11	16	c.1356delC	None
B22-1	Saudi Arabia	F	8	6	N/D	c.241G>T (D81Y)
B22-2	Saudi Arabia	F	13	12	N/D	c.241G>T (D81Y)
B23 (c)	Tunisian Jewish	M	11	22	c.1149+2T>G	N/D
B24 (c)	Iranian Jewish	F	13	16	c.89-90insG	N/D
B25	Iranian Jewish	M	8	5	c.89-90insG	N/D
B26-1	Italy	F	12	12	c.2T>C	None
B26-2	Italy	M	11	18	c.2T>C	None
B27-1	Italy	F	11	9	None	c.149+5G>A
B27-2	Italy	M	16	11	None	c.149+5G>A
B28	Italy	M	15	29	No detectable protein	None

All mutations are homozygous in affected individuals, except B28 whose mutation has not been identified.
(c) indicates known consanguinity; N/D, not done.

used to specifically identify the missense mutant proteins. All 3 missense mutants failed to coimmunoprecipitate with LMAN1 (Figure 1A). In contrast, the wild-type MCFD2 (both the endogenous and the myc-tagged proteins) are readily immunoprecipitated in complex with LMAN1, as detected by both anti-LMAN1 and anti-MCFD2 antibodies (Figure 1A). By immunofluorescence staining, all 3 missense mutant MCFD2 proteins are detected by anti-*myc* antibody in transfected cells, but fail to colocalize with LMAN1 (Figure 1B), in contrast to wild-type MCFD2. The staining pattern for all 3 missense MCFD2 mutations resembles that of protein disulfide isomerase (PDI), an ER marker (Figure 1B), similar to the pattern previously observed for the D129E and I136T mutations.[3]

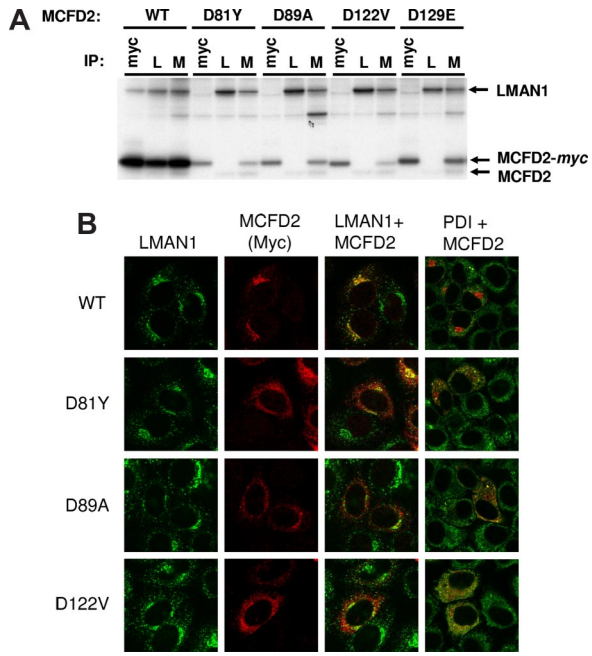

Figure 1. Characterization of MCFD2 missense mutations. (A) MCFD2 missense mutants fail to coimmunoprecipitate with LMAN1. COS1 cells were transfected with *myc*-tagged WT or mutant MCFD2, metabolically labeled, and immunoprecipitated with the indicated antibodies: myc indicates antimyc; L, anti-LMAN1; and M, anti-MCFD2. (B) MCFD2 missense mutants are mislocalized in cells. HeLa cells were transfected with *myc*-tagged WT or mutant MCFD2 and stained with rabbit anti-LMAN1, monoclonal antimyc, or monoclonal anti-PDI, and secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen, Carlsbad, CA).

Correlation of genotype with FV and FVIII levels

To date, at least 15 *MCFD2* mutations and 32 *LMAN1* mutations have been reported, including the 4 new mutations identified here. In addition, unidentified mutations may exist in regulatory regions of *LMAN1* that result in no mRNA accumulation.[6] Data on FV and FVIII levels are available for a total of 46 patients with *MCFD2* mutations and a total of 96 patients with *LMAN1* mutations (Table 2-3).[2-6,11,19-27] Figure 2 shows a comparison of FV and FVIII levels between patients with *MCFD2* mutations and patients with *LMAN1* mutations. We observed a small but statistically significant difference in the distribution of FV and FVIII levels between these 2 classes of patients, with lower levels for both factors in the patients with *MCFD2* mutations compared with those with *LMAN1* mutations (mean values of 9.6 vs 13.7 for FV [$P < .001$] and 10.0 vs 16.0 for FVIII [$P < .001$]). FVIII levels also exhibit a wider distribution, which is consistent with previous observations of healthy subjects and LMAN1 carriers.[28] In contrast, no significant differences were observed between male and female patients for either FV or FVIII levels (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). This includes analyses of the whole patient population, as well as subgroups divided by gene mutations *(LMAN1* versus *MCFD2)*. Although averages are slightly higher in all female groups, this trend is not statistically significant.

FVIII, but not FV, is known to be affected by ABO blood type, with lower levels in blood group O individuals thought to result from reduction in plasma von Willebrand factor.[29,30] We thus tested the possibility that differences in ABO blood group might partially explain the distribution of FVIII levels observed in Figure 2. For all patients listed in Tables 2-3 for whom DNA samples were available, genotyping was performed for the 2 most common O genotypes (O1 and O2, together accounting for $> 99\%$ of group O[31]) to distinguish blood group O from non-O types. No significant difference in FV and FVIII levels was observed in patients with blood group O compared with non-O blood groups (Figure S2).

Correlation of FV levels with FVIII levels

If the observed variation in factor levels is the result of biologic differences in the effect of loss of function for MCFD2 versus LMAN1, then plasma FV levels might be expected to correlate with FVIII levels for each individual patient. To test this hypothesis, we performed a Pearson correlation analysis on the FV/FVIII

Table 2. All reported F5F8D patients with *MCFD2* mutations, their geographic origins, sex, and FV/FVIII levels

	Patient	Origin	Sex	ABO	FV	FVIII	Mutation	Mutation effect	Ref no.
1	B3/5	Italy (c)	M	Non-O	2.3	6.3	c.149+5G>A	Splicing defect	5, 6
2	B4-1/6	Italy	M	O	7.5	6	c.149+5G>A	Splicing defect	5, 6
3	B4-2/6	Italy	F		10	10	c.149+5G>A	Splicing defect	5, 6
4	B5/8	Italy	F	Non-O	11	23	c.149+5G>A	Splicing defect	5, 6
5	B10	Serbia	F	O	7	7	c.149+5G>A	Splicing defect	5, 6
6	Zhang10-1/19	United States	M	Non-O	4	12	c.149+5G>A	Splicing defect	3
7	Zhang10-2/19	United States	M	Non-O	8	14	c.149+5G>A	Splicing defect	3
8	Zhang12-1/A32	Swiss	M	Non-O	7	10	c.149+5G>A	Splicing defect	3, 25
9	Zhang12-2/A32	Swiss	M		8	9	c.149+5G>A	Splicing defect	3, 25
10	Mohanty-2a	India	M		5	1.2	c.149+5G>A	Splicing defect	23
11	Mohanty-2b	India	F		6.6	4	c.149+5G>A	Splicing defect	23
12	Zhang1-4/A5	Iran	F	Non-O	5	10	c.309+1G>A	Splicing defect	3, 4
13	Zhang5-1/16	Turkey (c)	M	Non-O	7.5	10	c.309+1G>A	Splicing defect	3, 5
14	Zhang5-2/16	Turkey (c)	M	Non-O	7	7	c.309+1G>A	Splicing defect	3, 5
15	Zhang5-4/16	Turkey (c)	F	Non-O	10	8	c.309+1G>A	Splicing defect	3, 5
16	Zhang6-3	Turkey (c)	F	Non-O	9.5	9	c.249delT	Frameshift	3
17	Zhang6-4	Turkey (c)	M	Non-O	9.5	9	c.249delT	Frameshift	3
18	Zhang6-6	Turkey (c)	M	Non-O	9.5	9	c.249delT	Frameshift	3
19	B9	Kosovo	F	Non-O	4.5	1.5	c.407T>C	p.I136T	6
20	Zhang9-1/14	Venezuela	M	O	9.4	9	c.407T>C	p.I136T	3, 5
21	Zhang9-2/14	Venezuela	F	O	4.3	7.4	c.407T>C	p.I136T	3, 5
22	Zhang9-5/14	Venezuela	F	O	6.2	8.2	c.407T>C	p.I136T	3, 5
23	Zhang4-1/13	Venezuela	M	Non-O	9	7	c.387C>G	p.D129E	3, 5
24	Zhang2-1/A7	Iran (c)	M	O	7	7	unknown	Unknown	3, 4
25	B6/A14-III3	Iran (c)	M		10	6	c.-6-1G>A	Splicing defect	6, 24
26	B6/A14-III4	Iran (c)	F		10	13.5	c.-6-1G>A	Splicing defect	6, 24
27	B6/A14-III8	Iran (c)	F		10	6	c.-6-1G>A	Splicing defect	6, 24
28	B6/A14-III16	Iran (c)	F		10	21	c.-6-1G>A	Splicing defect	6, 24
29	Zhang7-1/A21	Italy	M	O	N/A	N/A	c.103delC	Frameshift	3, 4
30	Zhang7-2/A21	Italy	M	O	10	11	c.103delC	Frameshift	3, 4
31	Zhang11/A29	S. Africa	F	O	11	22	c.263-270del8nt	Frameshift	3, 4
32	India-1	India (c)	F		12.6	13.7	c.365A>C	p.D122V	11
33	India-2	India (c)	M		19.3	8.3	c.149+5G>A	Splicing defect	11
34	India-4	India (c)	F		22.4	27.1	c.149+5G>A	Splicing defect	11
35	India-5	India	M		14.1	12.3	c.149+5G>A	Splicing defect	11
36	India-6	India (c)	F		13.5	10.7	c.149+5G>A	Splicing defect	11
37	India-7	India (c)	F		5.6	7.8	c.149+5G>A	Splicing defect	11
38	India-8	India (c)	M		18.9	7.2	c.211-244del	Frameshift	11
39	India-9	India (c)	F		16.2	16.7	c.211-244del	Frameshift	11
40	Nyfeler	S. America	F		11.5	12	c.[431C>G]+[del8.5kb]	p.S144X+ Promoter deletion	27
41	B18	Greece	F	Non-O	7	5	c.266A>C	p.D89A	6
42	B19	Afro-Caribbean	F	Non-O	4	9	c.374-375insGA	Frameshift	This study
43	B22-1	Saudi Arabia	F	O	8	6	c.241G>T	p.D81Y	This study
44	B22-2	Saudi Arabia	F	O	13	12	c.241G>T	p.D81Y	This study
45	B27-1	Italy	F		11	9	c.149+5G>A	Splicing defect	This study
46	B27-2	Italy	M		16	11	c.149+5G>A	Splicing defect	This study

In cases where 2 different numbers were used to designate the same patient/family in different papers, both are indicated in the second column (separated by a slash). ABO indicates ABO blood group; Ref, reference; (c), known consanguinity in the family; N/A, information not available.

levels. Moderate correlation was observed in both patients with *MCFD2* mutations ($r = 0.53$, $P = .001$) and those with *LMAN1* mutations ($r = 0.322$, $P = .001$; Figure 3), suggesting that deficiencies in LMAN1 or MCFD2 exert a similar impact on FV and FVIII. A nonparametric Spearman correlation analysis yielded similar results (data not shown).

Correlation between platelet and plasma FV

Circulating FV exists in both plasma and platelets. The platelet FV pool has been shown to originate from endocytosis of plasma FV in humans[18,32]; although in mice, platelet FV is derived exclusively from biosynthesis within the megakaryocytes.[33,34] To address the question of whether *LMAN1* and *MCFD2* mutations exert differential effects between these 2 pools, we measured platelet FV antigen levels in 4 patients with *LMAN1* mutations and 3 patients with *MCFD2* mutations, as well as 15 healthy controls. Platelet FV levels are reduced to the same extent as FV levels in plasma, for both the *LMAN1* group (15% of control for plasma and 13% for platelets) and the *MCFD2* group (8% of control for both plasma and platelets; Table 4).

Discussion

Here we report 4 *MCFD2* and 5 *LMAN1* mutations. The mutation in family B19 appears to be the result of a duplication event, with insertion of a GA dinucleotide in a region that normally contains a

Table 3. All reported F5F8D patients with *LMAN1* mutations, their geographic origins, sex, and FV/FVIII levels

	Patient	Origin	Sex	ABO	FV	FVIII	Mutation	Mutation effect	Ref no.
1	1	Japan	M	Non-O	12	18	c.604C>T	p.R202X	5
2	2	Japan	F	Non-O	13	12	c.422delC	Frameshift	5
3	4	Italy	F	O/O	4.6	9.5	c.2T>C	p.M1T	5
4	7-1	Italy	M	Non-O	8	16	c.2T>C	p.M1T	5
5	7-2	Italy	M	Non-O	1.7	22	c.2T>C	p.M1T	5
6	9	Italy	F	Non-O	15	15	c.2T>C	p.M1T	5
7	10	Italy	M	O/O	13	15	c.2T>C	p.M1T	5
8	12-1	Venezuela	F	O/O	4	8	c.720-735del16bp	Frameshift	5
9	12-2	Venezuela	F	O/O	16	6.6	c.720-735del16bp	Frameshift	5
10	12-3	Venezuela	F		7	10	c.720-735del16bp	Frameshift	5
11	12-4	Venezuela	F		40	20	c.720-735del16bp	Frameshift	5
12	12-5	Venezuela	M	O/O	17	10	c.720-735del16bp	Frameshift	5
13	15-1	France	F	Non-O	25	10	c.904A>T	p.K302X	5
14	15-2	France	F	O/O	5	20	c.904A>T	p.K302X	5
15	17	US Armenian	N/A		N/A	N/A	c.1519delA	Frameshift	5
16	18	United States	N/A		N/A	N/A	c.1109-1121delTC	Frameshift	5
17	B11-1	Italy	M	Non-O	26	23	c.2T>C	p.M1T	6
18	B11-2	Italy	F	Non-O	13	20	c.2T>C	p.M1T	6
19	A1-IV1	Iran (c)	M		8	5.5	c.912-913insA	Frameshift	4
20	A1-IV4	Iran (c)	F		7	14	c.912-913insA	Frameshift	4
21	A1-IV6	Iran (c)	F		11	9	c.912-913insA	Frameshift	4
22	A2-IV1	Iran (c)	F	Non-O	7.5	7	c.912-913insA	Frameshift	4
23	A3-IV4	Iran (c)	F	Non-O	20	16	c.[912-913insA] +[89-90insG]	Frameshift	4
24	A4-IV7	Iran (c)	M		10.5	11	c.1149+2T>G	Splicing defect	4
25	A6-III1	Iran (c)	F		12	12.5	c.822-1G>A	Splicing defect	4
26	A6-III2	Iran (c)	M		12	14	c.822-1G>A	Splicing defect	4
27	A8-III2	Iran (c)	M		5	6	c.1214-1218delAAATG	Frameshift	4
28	A9-III2	Iran (c)	M		18	8	c.822-1G>A	Splicing defect	4
29	A10-III1	Iran (c)	F		12	15	c.23delG	Frameshift	4
30	A10-III4	Iran (c)	M		19	16	c.23delG	Frameshift	4
31	A11-IV4	Iran (c)	M	O/O	18	17	c.89-90insG	Frameshift	4
32	A12-III3	Iran (c)	M	Non-O	2.5	2.2	c.822-1G>A	Splicing defect	4
33	A16-II7	Iran (c)	M		9	13.5	c.604C>T	p.R202X	4
34	A17-III1	Iran (c)	M		5	7.5	c.604C>T	p.R202X	4
35	A17-IV1	Iran (c)	F		34	14.5	c.604C>T	p.R202X	4
36	A17-IV2	Iran (c)	M		10	8	c.604C>T	p.R202X	4
37	A18-II3	Pakistan (c)	F	Non-O	10	15	c.904A>T	p.K302X	4
38	A19-II1	Pakistan (c)	F	Non-O	14	14	c.1366C>T	p.R456X	4
39	A19-II2	Pakistan (c)	M		14	14	c.1366C>T	p.R456X	4
40	A20-1	Pakistan (c)	M		8	6	c.904A>T	p.K302X	4
41	A20-2	Pakistan (c)	F		11	7.7	c.904A>T	p.K302X	4
42	A22 (B7)	Italy	M	Non-O	8	24	exon4 skipping†	no protein	4
43	A24	Italy	F	O/O	17	26	c.2T>C	p.M1T	4
44	A25-1	Italy	M	Non-O	9	10	c.2T>C	p.M1T	4
45	A25-2	Italy	F		N/A	N/A	c.2T>C	p.M1T	4
46	A26-1	Pakistan	M		18	18	c.904A>T	p.K302X	4
47	A26-2	Pakistan	M		18	18	c.904A>T	p.K302X	4
48	A27	China	M		7	9	c.1366C>T	p.R456X	4
49	A30	Pakistan (c)	F		6	3	c.904A>T	p.K302X	4
50	A31	Pakistan (c)	M		14	18	c.904A>T	p.K302X	4
51	A34	Italy	M		9	27	c.639+1G>T	Splicing defect	4
52	A35-1	Italy	F		6	23	c.639+1G>T	Splicing defect	4
53	A35-2	Italy	F		8	25	c.639+1G>T	Splicing defect	4
54	A36	Italy	M		14	27	c. 1208-1209insT	Frameshift	4
55	A37	China	F		17	19	c.1366C>T	p.R456X	4
56	B1	Algeria	M		N/A	N/A	c.31delG	Frameshift	4
57	B12-1	Austria	M	O/O	15	12	c.780delT	Frameshift	6
58	B12-2	Austria	F	O/O	24	13	c.780delT	Frameshift	6
59	B13	Iraq	F	O/O	14	25	c.961G>T	p.E321X	6

In cases where 2 different numbers were used to designate the same patient/family in different publications, both are indicated in the second column (separated by a slash). Additional clinical data on previously unreported siblings have been added into certain families. Patient A30 appears to also have von Willebrand disease, and therefore is excluded from the statistical analysis.

ABO indicates ABO blood group; Ref, reference; (c), known consanguinity in the family; and N/A indicates information not available.

*Causative relationship has not been established for this mutation/polymorphism.

†Compound heterozygote with no mutation detected on the second allele.

Table 3. All reported F5F8D patients with *LMAN1* mutations, their geographic origins, sex, and FV/FVIII levels (continued)

	Patient	Origin	Sex	ABO	FV	FVIII	Mutation	Mutation effect	Ref no.
60	B14	Poland (c)	M	O/O	16	23	c.839delA	Frameshift	6
61	B15	Belgium	N/A	Non-O	11	35	c.822+1G>A	Splicing defect	6
62	B16	United States	F	Non-O	23	24	c.822+33-34insGGTT*†	Exon 8 skipping	6
63	B17	Argentina	F	Non-O	18	23	c1423T>C†	p.C475R	6
64	Dansako-1	Japan	N/A		12	12	c.604C>T	p.R202X	19
65	Thai	Thailand	F		10	12.5	c.[1366C>T]+[823-1G>C]	p.R456X+splicing	26
66	India-3	India (c)	F		9.5	16.1	c.813-822 + 62del72	Frameshift	11
67	Farah	Lebanon	F		13.7	8	c.[1138C>T]+[1270delG]	p.Q380X+frameshift	21
68	Mohanty-3	India	F		7	2.2	c.340G>T	p.G114X	23
69	D′Ambrosio	Italy	M		8.7	13.7	c.2T>C	p.M1T	19
70	Nichols-1	Tunisian Jewish (c)	M		5.7	13.7	c.1149+2T>G	Splicing defect	2
71	Nichols-5a	Tunisian Jewish (c)	F		11.7	23.7	c.1149+2T>G	Splicing defect	2
72	Nichols-5b	Tunisian Jewish (c)	M		23	22	c.1149+2T>G	Splicing defect	2
73	Nichols-6	Tunisian Jewish (c)	F		9	24	c.1149+2T>G	Splicing defect	2
74	Nichols-8	Tunisian Jewish (c)	M		14.5	9.5	c.1149+2T>G	Splicing defect	2
75	Nichols-9a	Tunisian Jewish (c)	F		14.3	17.2	c.1149+2T>G	Splicing defect	2
76	Nichols-9b	Tunisian Jewish (c)	M		20	32	c.1149+2T>G	Splicing defect	2
77	Nichols-2a	Iraqi Jewish (c)	F		23	10	c.89-90insG	Frameshift	2
78	Nichols-2b	Iraqi Jewish (c)	F		23	13	c.89-90insG	Frameshift	2
79	Nichols-2c	Iraqi Jewish (c)	F		14	13	c.89-90insG	Frameshift	2
80	Nichols-2d	Iraqi Jewish (c)	F		28	46	c.89-90insG	Frameshift	2
81	Nichols-2e	Iraqi Jewish (c)	M		24	24.5	c.89-90insG	Frameshift	2
82	Nichols-3a	Iranian Jewish (c)	F		13.7	19.3	c.89-90insG	Frameshift	2
83	Nichols-3b	Iranian Jewish (c)	M		23	22	c.89-90insG	Frameshift	2
84	11	Iranian Jewish	F	Non-O	8.3	21.7	c.89-90insG	Frameshift	5
85	Nichols-4a	Iraqi Jewish (c)	M		11	19	c.89-90insG	Frameshift	2
86	Nichols-4b	Iraqi Jewish (c)	F		15	14	c.89-90insG	Frameshift	2
87	Nichols-7a	Egyptian Jewish (c)	F		12.8	15.6	c.89-90insG	Frameshift	2
88	Nichols-7b	Egyptian Jewish (c)	M		10.4	25.25	c.89-90insG	Frameshift	2
89	Ma-1	China	M		12.4	8.2	c.[949C>T]+[1366C>T]	p.[Q317X]+[R456X]	22
90	Ma-2	China	F		13.5	11.3	c.[949C>T]+[1366C>T]	p.[Q317X]+[R456X]	22
91	B21-1	Turkey (c)	M	Non-O	35	15	c.795delC	Frameshift	This study
92	B21-2	Turkey (c)	M	Non-O	25	20	c.795delC	Frameshift	This study
93	B22-1	Iraq	F	Non-O	12	18	c.1356delC	Frameshift	This study
94	B22-2	Iraq	M	Non-O	11	16	c.1356delC	Frameshift	This study
95	B23	Tunisian Jewish (c)	M	Non-O	11	22	c.1149+2T>G	Splicing defect	This study
96	B24	Iranian Jewish (c)	F	Non-O	13	16	c.89-90insG	Frameshift	This study
97	B25	Iranian Jewish	M	Non-O	8	5	c.89-90insG	Frameshift	This study
98	B28-1	Italy	F	Non-O	12	12	c.2T>C	M1T	This study
99	B28-2	Italy	M	Non-O	12	12	c.2T>C	M1T	This study
100	B29	Italy	M		15	29	Unknown	No protein	This study

In cases where 2 different numbers were used to designate the same patient/family in different publications, both are indicated in the second column (separated by a slash). Additional clinical data on previously unreported siblings have been added into certain families. Patient A30 appears to also have von Willebrand disease, and therefore is excluded from the statistical analysis.

ABO indicates ABO blood group; Ref, reference; (c), known consanguinity in the family; and N/A indicates information not available.

*Causative relationship has not been established for this mutation/polymorphism.

†Compound heterozygote with no mutation detected on the second allele.

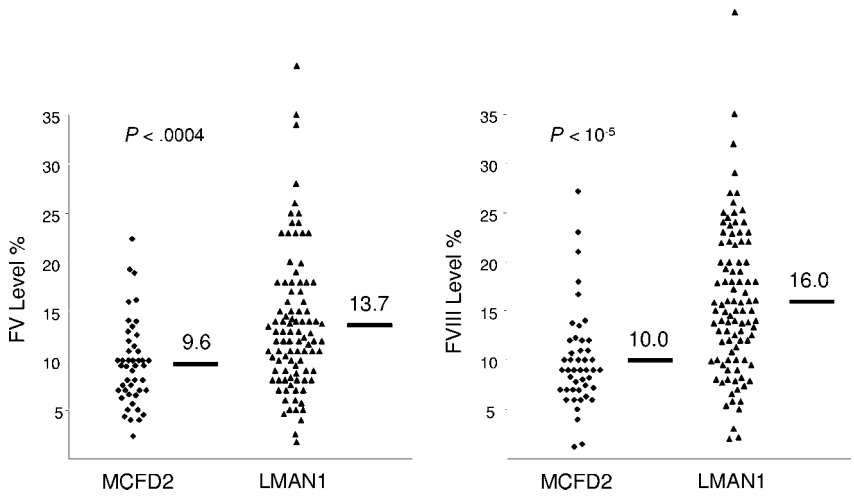

Figure 2. Correlation of genotypes and levels of FV and FVIII in F5F8D patients. Distribution of FV levels and FVIII levels in patients with *LMAN1* mutations and *MCFD2* mutations. The short bars and numbers indicate the average values. To meet the assumptions of normality, before the Student *t* test analysis, the FV and FVII data were cube-root transformed.

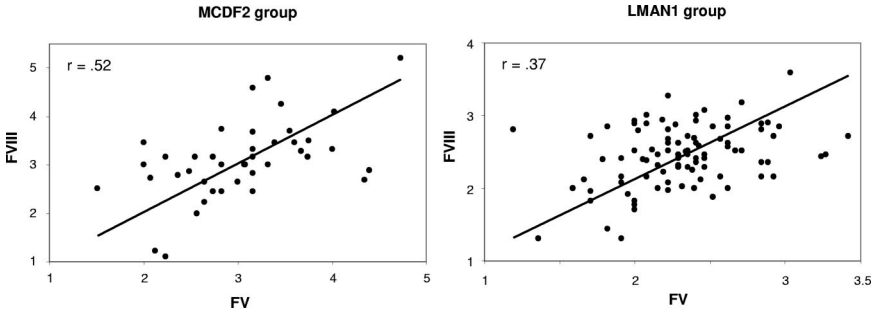

Figure 3. Correlation of FV levels and FVIII levels. Shown are Pearson correlation analysis on FV and FVIII levels (cube-root transformed) for each individual patient in (A) patients with *MCFD2* mutations ($P = .001$) and (B) patients with *LMAN1* mutations ($P < .001$).

string of 3 GAs. This mutation results in a frameshift that deletes both EF hand domains of MCFD2. The mutation in family B22 (D81Y) is the fifth missense mutation identified in MCFD2, and the second identified in the first EF hand domain. In the current report, we demonstrate that this mutation, as well as 2 other missense mutations identified previously, disrupts the binding to LMAN1 (Figure 1). Taken together with results of previously reported D129E and I136T mutations, all missense mutations identified to date are located in the EF hand domains and result in loss of LMAN1 binding. The 2 mutations identified in families B23 to B25 are consistent with the previously reported founder effect.[2] To date, all F5F8D patients in Jewish families have been found to carry either the c.1149+2T>G (Tunisian Jews) or the c.89-90insG mutation (Middle Eastern Jews), with the combined prevalence of one of these mutations in these populations estimated at 1:100 000.[12,28] The c.2T>C mutation in *LMAN1* identified in family B26 has been reported only in patients of Italian origin; therefore, it likely represents a founder allele in the Italian population. The mutation in family B27 (c.149+5G>A) is thus far the most frequently identified mutation in *MCFD2*. Previous studies have shown that this is a recurring mutation that arose independently in different geographic regions.[6,11]

The difference observed between LMAN1 and MCFD2 patients in the distribution of plasma levels for FV and FVIII is unlikely due to a systematic error in measurement as these data were collected at multiple centers around the world. However, a consistent trend toward high or low values from one of the centers contributing a relatively large group of patients could potentially bias the results,

Table 4. FV levels in plasma and platelets, the means and the ratios between patients and healthy controls.

Patient	Plate count, $\times 10^6$ plts/mL	Plasma FV, ng/mL	Plasma FV, ng/mL blood	Platelet FV, ng/10^9 plts	Platelet FV, ng/mL blood	Platelet FV/ total FV, %
Control group						
Control 1	427	7242	4251	778	332	7.2
Control 2	331	8000	4424	661	219	4.7
Control 3	186	6708	3542	545	101	2.9
Control 4	319	10600	5183	670	214	4.0
Control 5	246	9100	5305	461	113	2.1
Control 6	218	5950	3403	860	187	5.2
Control 7	286	7300	4190	294	84	2.0
Control 8	187	7733	3952	536	100	2.5
Control 9	324	8205	4201	654	212	6.6
Control 10	232	8845	5316	507	118	2.8
Control 11	267	10120	5991	820	219	2.0
Control 12	288	7497	4506	579	167	3.6
Control 13	205	7250	4401	681	140	3.1
Control 14	254	12129	6174	864	219	3.4
Control 15	239	8329	4389	596	142	3.1
Mean (±SD)	267 (±65)	8334 (±1615)	4615 (±815)	634 (±158)	171 (±67)	3.7 (±1.6)
LMAN1 group						
B26-1	210	1050	609	75	16	2.5
B26-2	130	789	633	62	13	2.6
B28	211	1150	447	128	17	2.8
A24	258	1850	1073	172	44	4.0
Mean (±SD)	202 (±53)	1210 (±453)	690 (±268)	109 (±51)	23 (±15)	3.0 (±0.7)
MCFD2 group						
B27-1	193	570	335	54	10	3.0
B27-2	238	790	466	67	16	3.3
Zhang7-2	206	580	319	54	11	3.4
Mean (±SD)	212 (±23)	647 (±124)	373 (±81)	58 (±7)	13 (±3)	3.2 (±0.2)
Ratios						
LMAN1/Wt.			0.15		0.13	
MCFD2/Wt.			0.08		0.08	

Plasma and platelet FV antigen levels were measured by ELISA. Plasma FV and platelet FV were normalized to the hematocrit and platelet counts, respectively, to determine the amount of FV of each pool in whole blood.

plts indicates platelets.

particularly given the smaller sample size for the *MCFD2* group. Of note, most of the patients in a recent report from India (included in our analysis) have *MCFD2* mutations,[11] with measurements clustered at the high end of the range for both FV and FVIII. However, if data from these patients are removed, the difference in levels between the *MCFD2* and *LMAN1* groups increases even further in significance (mean values of 8.4 vs 13.7 for FV [$P < .001$] and 9.3 vs 16.0 for FVIII [$P < .001$]). A recent report of an additional 9 F5F8D patients from 5 families is notable for levels in the lower end of the range, particularly for FVIII (at 1% or lower for 6 of 9 patients).[35] Mutations have not been identified in these patients. In addition to laboratory-specific bias in factor activity measurements and biologic differences between *MCFD2* and *LMAN1,* other factors may contribute to variations in plasma FV and FVIII levels among certain groups of patients, including common genetic modifiers or environmental factors unique to the corresponding population. However, sex and ABO blood type do not appear to be important modifying factors (Figure 3). Interestingly, FVIII levels in F5F8D patients with type O blood are not further reduced compared with non-O blood types.

Platelet FV levels are reduced to the same extent as plasma FV levels, for both the *LMAN1* group and the *MCFD2* group (Table 4). A prior study on 2 F5F8D patients is consistent with our results, demonstrating reduced factor Xa binding in platelet releasates to approximately 40% of the healthy control.[36] The platelet FV pool in humans is derived via endocytosis of plasma FV,[18,32] whereas in the mouse, platelet FV is synthesized de novo in the megakaryocytes.[33,34] Recent studies suggest that endocytosis of FV is a specific, clathrin-dependent, and probably receptor-mediated mechanism.[37] Our observations of similarly reduced FV levels in platelets and plasma of F5F8D patients suggest that the receptor for uptake of plasma FV into megakaryocytes is not saturated at physiologic concentrations of plasma FV. Alternatively, a mechanism may exist that regulates the rate of FV uptake into megakaryocytes to match the plasma FV level. Consistent with either of these models, the amount of intracellular FV in cultured megakaryocytes has been shown to be dependent on the exogenous factor V concentrations in the culture media.[38] Our results would predict that other conditions that alter plasma FV level should also alter the platelet level, including severe liver diseases or autoimmune clearance of plasma FV. Although a number of patients with FV inhibitor antibodies have been described,[39-42] bleeding symptoms are highly variable, and direct measurement of platelet FV antigen in this setting has not been reported.

The finding that *MCFD2* mutations are generally associated with lower levels of FV and FVIII suggests that MCFD2 may play a more direct role in transporting FV and FVIII, perhaps initiating the interaction with these 2 cargo proteins in the ER and/or recruiting them to ER exit sites. Consistent with this hypothesis, MCFD2 with a missense mutation (D129E) that disrupts the LMAN1-MCFD2 interaction can still be cross-linked to FVIII.[10] The lectin activity of LMAN1 may function to further stabilize the receptor-cargo complex by binding to the sugar residues of FV and FVIII. Alternatively, LMAN1 may merely function to carry MCFD2 and FV/FVIII cargo to the ER exit sites for packaging into COPII vesicles. In this model, LMAN1 deficiency would act only through the resulting secondary MCFD2 deficiency and higher levels of FV/FVIII in *LMAN1*$^{-/-}$ patients could reflect FV/FVIII transport by the small, residual pool of intracellular MCFD2. A recent study suggests that MCFD2 is dispensable for the interaction of LMAN1 with the lysosomal enzymes cathepsin C and cathepsin Z.[43] Thus, MCFD2 could function as a selective cargo adaptor for FV and FVIII, with LMAN1 deficiency resulting in subclinical deficiencies for additional cargo proteins that directly interact with LMAN1 or are linked to adaptors other than MCFD2. This hypothesis awaits experimental confirmation.

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Authorship

Contribution: B.Z., M.S., C.Z., and A.Y. performed experiments; B.Z., F.P., and D.G. designed the research and analyzed results; B.Z. made the figures; P.P. performed statistical analyses; B.Z., M.S., F.P., and D.G. wrote the paper; other authors provided vital new reagents and clinical data, and critical review of the paper.

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