Author contributions

N.Y. and K.K. designed and performed the research and wrote the paper; N.Y., H.S., K.M., and K.K. collected and managed clinical data.

Disclosure of conflicts of interest

The authors declare no conflicts of interest.

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Two novel mutations in the *tmprss6* gene associated with iron-refractory iron-deficiency anaemia (irida) and partial expression in the heterozygous form

Iron-refractory iron-deficiency anaemia (IRIDA, Online Mendelian Inheritance in Man number 206200) is an autosomal recessive genetic disorder characterized by iron deficiency anaemia unresponsive to oral iron treatment but partially responsive to parenteral iron therapy (Finberg, 2009). IRIDA is due to mutations in the *TMPRSS6* gene, which encodes the serine-protease matriptase 2 (Finberg *et al*, 2008), an inhibitor of the iron-related hormone, hepcidin. Several mutations in the *TMPRSS6* gene have been characterized in IRIDA families of different ethnic origins. Recent

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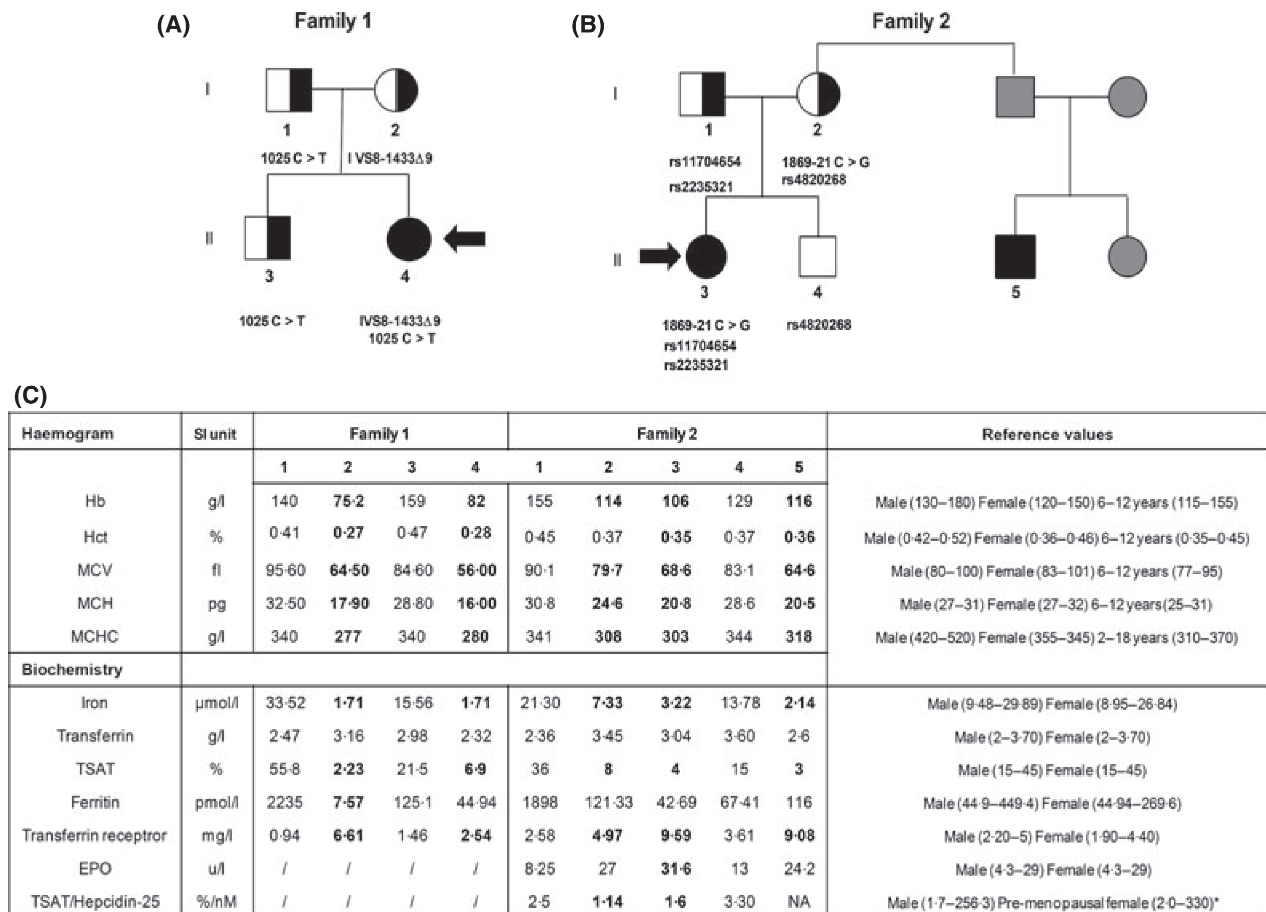
findings on *Tmprss6*-haploinsufficient mice support the hypothesis that susceptibility to iron deficiency may be increased by the presence of *TMPRSS6* mutations even in the heterozygous state (Nai *et al*, 2010). Here we present two new *TMPRSS6* variants that were associated, in the heterozygous form, with manifest IRIDA in two un-related families.

In the Italian Family 1, the proband was a 9-year-old girl with microcytic anaemia, low serum iron and normal ferritin (Fig 1A, C). She had no response to iron oral therapy but she responded to i.v. iron therapy, reaching haemoglobin values stably higher than 110 g/l with ferritin values around 440 pmol/l. The molecular study of family members revealed the absence of *HBA1*/*HBA2* and *HBB* gene defects. The proband's mother also presented blood parameters consistent with iron-deficient anaemia. She referred mild hypermenorrhea but was negative for the most common malabsorption causes. She was treated with oral iron, with poor results (haemoglobin 75 g/l vs. 91 g/l after 5 months oral iron therapy).

In the Portuguese Family 2, a 9-year-old girl was diagnosed with microcytic anaemia, low serum iron and normal ferritin (Fig 1B, C). After no response to oral iron therapy, she started with i.v. iron, with no response (haemoglobin increase 5 g/l, with no reticulocytosis). Ferritin increased significantly (360 pmol/l) but iron remained low. Specific tests excluded other causes of iron-deficiency anaemia, such as malabsorption and bleeding, and the common alpha/beta thalassemia mutations. Her mother had a non-symptomatic mycrocitic and hypochromic anaemia (Fig 1C). Again, specific tests excluded secondary causes of iron deficient anaemia or thalassemia trait (including common alpha/beta thalassemia mutations). The father had hyperferritinaemia, probably due to heavy alcohol consumption. A microcytic anaemia refractory to oral iron therapy was also documented in a 6-year-old maternal cousin. Serum hepcidin-25 values



Haemogram	SI unit	Family 1				Family 2					Reference values
		1	2	3	4	1	2	3	4	5	
Hb	g/l	140	**75·2**	159	**82**	155	**114**	**106**	129	**116**	Male (130–180) Female (120–150) 6–12 years (115–155)
Hct	%	0·41	**0·27**	0·47	**0·28**	0·45	0·37	**0·35**	0·37	**0·36**	Male (0·42–0·52) Female (0·36–0·46) 6–12 years (0·35–0·45)
MCV	fl	95·60	**64·50**	84·60	**56·00**	90·1	**79·7**	**68·6**	83·1	**64·6**	Male (80–100) Female (83–101) 6–12 years (77–95)
MCH	pg	32·50	**17·90**	28·80	**16·00**	30·8	**24·6**	**20·8**	28·6	**20·5**	Male (27–31) Female (27–32) 6–12 years(25–31)
MCHC	g/l	340	**277**	340	**280**	341	**308**	**303**	344	**318**	Male (420–520) Female (355–345) 2–18 years (310–370)
Biochemistry											
Iron	μmol/l	33·52	**1·71**	15·56	**1·71**	21·30	**7·33**	**3·22**	13·78	**2·14**	Male (9·48–29·89) Female (8·95–26·84)
Transferrin	g/l	2·47	3·16	2·98	2·32	2·36	3·45	3·04	3·60	2·6	Male (2–3·70) Female (2–3·70)
TSAT	%	55·8	**2·23**	21·5	**6·9**	36	**8**	**4**	15	**3**	Male (15–45) Female (15–45)
Ferritin	pmol/l	2235	**7·57**	125·1	44·94	1898	121·33	42·69	67·41	116	Male (44·9–449·4) Female (44·94–269·6)
Transferrin receptror	mg/l	0·94	**6·61**	1·46	**2·54**	2·58	**4·97**	**9·59**	3·61	**9·08**	Male (2·20–5) Female (1·90–4·40)
EPO	u/l	/	/	/	/	8·25	27	**31·6**	13	24·2	Male (4·3–29) Female (4·3–29)
TSAT/Hepcidin-25	%/nM	/	/	/	/	2·5	**1·14**	**1·6**	3·30	NA	Male (1·7–256·3) Pre-menopausal female (2·0–330)*

Fig 1. (A, B) IRIDA Family trees with single member genotypes. Arrows indicate probands. Subjects unavailable for the analysis are shown in grey. (C) Haematological and biochemical parameters for the two families. Hb: haemoglobin; Hct: haematocrit; MCV: mean cell volume; MCH: mean cell haemoglobin; MHC: mean haemoglobin concentration; TSAT: transferrin saturation; EPO: erythropoietin; (TSAT)/Hepcidin-25: Transferrin saturation/serum hepcidin-25 ratio. Reference values are reported on the right. Values outside normal ranges are indicated in bold. *A low transferrin saturation (TSAT)/Hepcidin-25 ratio may be consistent with Iron Refractory Iron Deficiency Anaemia (IRIDA), (http://www.hepcidinanalysis.com).

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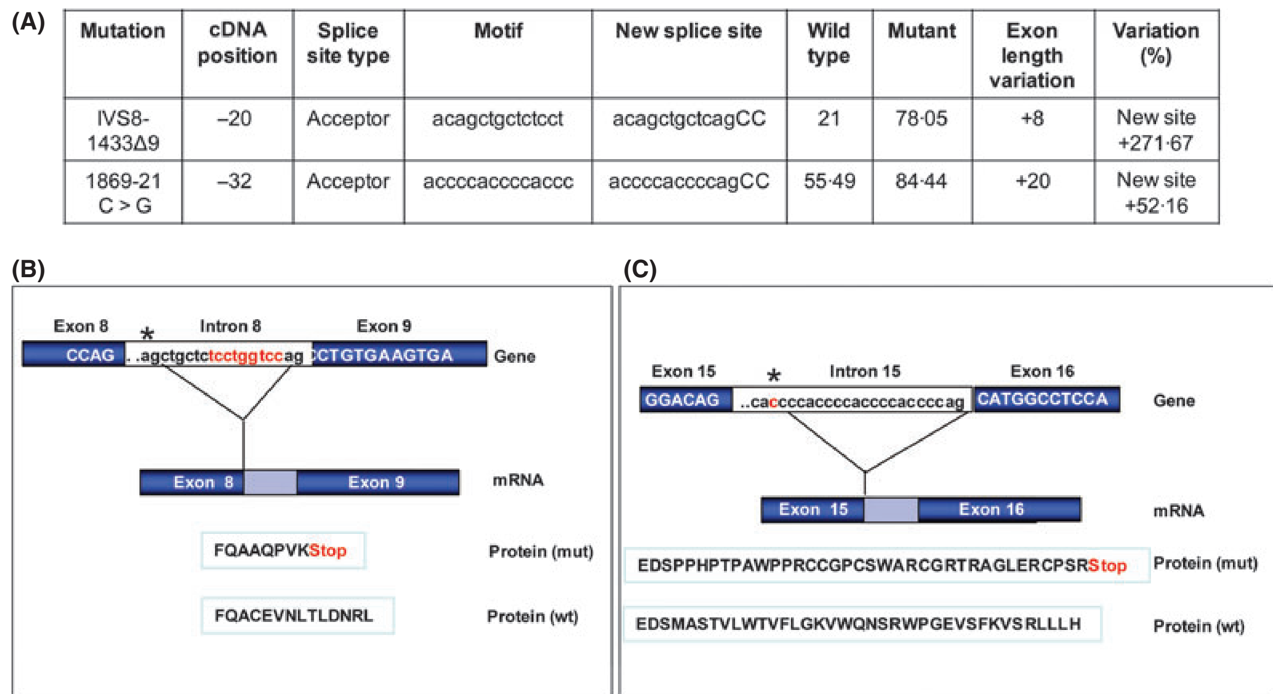

(A)

Mutation	cDNA position	Splice site type	Motif	New splice site	Wild type	Mutant	Exon length variation	Variation (%)
IVS8-1433Δ9	−20	Acceptor	acagctgctctcct	acagctgctcagCC	21	78·05	+8	New site +271·67
1869-21 C > G	−32	Acceptor	accccaccccaccc	accccaccccagCC	55·49	84·44	+20	New site +52·16

Fig 2. (A) Prediction of creation of potential splicing sites in mutated *TMPRSS6* sequences, using Human Splicing Finder. (B) Schematic representation of the effects of IVS8-1433Δ9 and (C) *c.* 1869-21 C>G mutations on mRNA processing. Capital letters in the gene schematics indicate transcribed nucleotides. Deleted/mutated nucleotides are in red, cryptic acceptor splice sites are marked by asterisks, dotted areas show the added transcribed sequences.

and serum transferrin saturation/hepcidin-25 ratio, a marker for IRIDA, showed values below the reference for both the proband and her mother (Fig 1C).

After all family members provided informed consent, DNA was extracted from peripheral blood. *TMPRSS6* exons and exon-intron boundaries were amplified and automatically sequenced. *HBA1*/*HBA2* and *HBB* molecular analysis was performed with a reverse dot blot kit (Nuclear Laser Medicine Strip Assay, Italy). The effect of mutations on *TMPRSS6* mRNA maturation was predicted utilizing the ALAMUT (Interactive Biosoftware, Rouen, France) and the Human Splicing Finder (Desmet *et al*, 2009) software. For expression analysis of mutated transcripts, total RNA was extracted from peripheral blood mononuclear cells, reverse-transcribed and amplified by qualitative or quantitative real time polymerase chain reaction (PCR).

A previously reported mutation in exon 8 (1025 C>T, S304L) (De Falco *et al*, 2010) was found in heterozygosity in Family 1 proband (Fig S1), which was also present in her father and brother (Fig 1A). On the other patient's allele, a 9 bp deletion was identified in heterozygous form at the end of intron 8 (IVS8-1433 Δ9) (Fig S1), which was inherited from her mother (Fig 1A). *In silico* analysis predicted that the deletion abolishes the constitutive splice site and favours the use of a cryptic site 17 bp upstream (Fig 2A). As a consequence of this, 8 bp are added to the coding sequence, causing a frameshift and the creation of a premature stop codon (Fig 2B). Real time PCR analysis revealed the presence of two mRNA forms in the proband, one of which was found at lower amounts compared to the other (Fig S2). This is probably due to the faster degradation of the mutated mRNA in comparison to the wild-type.

A heterozygous mutation at the end of intron 15 (*c.* 1869-21 C>G) was identified in the proband from Family 2 (Fig S1), which was inherited from her mother (Fig 1B). Two synonymous polymorphisms (rs11704654 and rs2235321, corresponding to Pro32 and Tyr738 respectively), previously associated with IRIDA (Delbini *et al*, 2010) were also found, both of which were also present in the proband's father. *In silico* analysis of the effect of the *c.* 1869-21 C>G mutation predicted the creation of a new splicing site, approximately 50% stronger than the native site, localized 21 bp downstream (Fig 2A). The mutated mRNA would contain an extra 20 bp, causing a frameshift and the creation of a premature stop codon. As a consequence, the new protein would lack 96% of the serine protease domain (Fig 2C). Analysis of mRNA expression using primers amplifying the mutated locus showed the presence of two mRNA forms differing by approximately 20 bp, while amplification with primers specific for the mutation was observed only for the proband and her mother (Fig S2).

The characterization of new mutations in the *TMPRSS6* gene in two young patients with IRIDA further confirms the allelic heterogeneity of this disorder. In Family 1, the

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presence of the IVS8-1433Δ9 variation at the heterozygous state in the mother was associated with a consistent anaemia that was resistant to oral iron therapy and not completely explainable by secondary causes. In Family 2, the new 1869-21 C>G mutation is associated with two iron deficiency-related polymorphisms both in the proband and her symptomatic cousin. The two polymorphisms alone cannot be accountable for the disease, as these are both present in the proband's asymptomatic father. Thus, it is apparent that the development of IRIDA symptoms in Family 2 needs the presence of the 1869-21 C>G mutation. Curiously, the proband's mother, who carries the 1869-21 C>G mutation, but not the two polymorphisms, although asymptomatic, has a picture of mild iron-deficient anaemia.

In conclusion, the data suggest that although heterozygous *TMPRSS6* mutations may not be able to induce a clear IRIDA phenotype, some of them may increase the susceptibility to iron deficiency.

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Authorship contribution

RMP and MC collected clinical data and performed the molecular analysis, DDA, GZ; E Costa and JB referred patients for molecular diagnosis; AML, AP, MC and FC performed DNA and RNA extraction and sequencing, GP, JPP and AR coordinated experiments and wrote the paper, GS read the manuscript and added critical suggestions.

Conflict of interest

All the authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Electrophoretograms of the *TMPRSS6* genomic sequences spanning the mutations found in Family 1 (a) and in Family 2 (b) patients. The DNA sequences of a wild type subject (WT) and of the probands (P) are shown. Mutations are indicated by asterisks.

Fig S2. (a) RT-PCR melting curves of Family 1 father (I-1, upper panel) and proband (II-4, middle panel) *TMPRSS6* transcripts. Abelson Tyrosine-kinase (*ABL*) cDNA amplification, used as calibrator, is shown in the bottom panel. The pink line represents the established threshold. (b) Effects of the *c.* 1869-21C>G mutation on *TMPRSS6* mRNA. Upper panel: Alternative spliced cDNA created by the mutation electrophoresed on agarose gel. cDNAs including the mutated loci (1 and 2) or specific for the mutated sequence (3) were obtained and amplified for the proband (P), mother (M) and father (F) and electrophoresed. Two bands, with molecular weights in agreement with the predicted WT (404 and 158 bp, respectively for amplicons 1 and 2) or mutant (424 and 178 bp, respectively for amplicons 1 and 2) sizes were observed for proband and mother, whereas only the smaller band was observed for the father. A band corresponding to the mutated sequence (140 bp) was only observed in the proband and mother. L: 1Kb DNA Plus Ladder. Bottom panel: Map of the expected amplicons for WT and mutated regions.

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Food allergy after cord blood transplantation in children

A significant number of factors play an important role in the development of food allergy (FA), including early introduction of solid food, genetically determined bias toward a T helper cell type 2 (Th2) environment, and polymorphisms of Th2 cytokine and immunoglobulin E (IgE) receptor genes. While most food allergies are IgE-mediated, a number of non-IgE-mediated gastrointestinal FA affect mainly infants and young children (Sicherer & Sampson, 2010). Allogeneic haematopoietic stem cell transplantation (HSCT) is an optimal treatment for haematological disease. Agosti *et al* (1988) reported transfer of allergen-specific IgE-mediated hypersensitivity with allogeneic bone marrow transplantation (BMT). The passive transfer of B- and/or helper T-cell clones with allergen-specific memory within the BM inoculum is a possible mechanism.

Here a chart review was performed to conduct a retrospective analysis of the development of FA among 14 children (0·4–13 years) who survived for more than 1 year after cord blood transplantation (CBT) between 1998 and 2011 at our institute. The study protocol was approved by our institutional review board. Four patients were transplanted with fully human leucocyte antigen (HLA)-matched unrelated CB cells, and nine cases were from HLA-mismatched unrelated donors after total body irradiation (6–12 Gy)-containing regimen. Twelve patients received tacrolimus (FK506) and methylprednisolone as graft-*versus*-host disease (GVHD) prophylaxis. One patient received FK506 and short-term methotrexate, and the remaining patient was given cyclosporine (CsA) and short-term methotrexate. After engraftment, the dose of methylprednisolone was tapered and discontinued up to day 30 in the absence of acute GVHD. At 3–6 months following CBT, five children developed urticaria, angioedema, diarrhoea and vomiting, alone or in combination immediately after the ingestion of one or more foods (soybean, milk, cow's milk, egg, rice, etc., Table I). The five patients had neither past history nor family history of allergic disorders before CBT. Mean age at CBT was 1·6 ± 1·3 years in five patients with the symptoms described above and 5·6 ± 4·5 years in nine patients without the symptoms ($P = 0{\cdot}06$). At the appearance of the symptom(s), four patients had been treated with FK506 with or without methylpredonisolone and the remaining patient with CsA because of the development of acute GVHD. Their FK506 and CsA blood levels were 4·5–10·6 and 98–132 ng/ml, respectively. Avoidance of the suspected food(s) resolved the symptoms in all five patients. Taking these findings together, we made the diagnosis of post-CBT FA. At the occurrence of FA symptoms, total IgE levels were not high in all the patients except for Patient 5. Total IgE levels reached the maximum (higher than 3000 iu/ml) at 9 and 26 months after the onset of FA, in Patients 1 and 2, respectively. Additionally, specific IgE tests revealed apparent positivity against multiple foods. No increases in specific IgE were noted during the clinical course in the other three patients. Patient 1 had specific IgE against caseins, alpha-lactalbumin, and beta-lactoglobulin, at 50·9, 0·42 and 1·16 allergen units (au)/ml, respectively. Skin prick tests were not performed. Reproducibility of the allergic reaction was found' by allergen challenge in four patients. Colonic biopsy revealed chronic inflammation with modestly increased eosinophil infiltration in the lamina propria of terminal ileum and colon in Patients 4 and 5. The characteristic findings of cord colitis syndrome described by Herrera *et al* (2011) were not identified. Thus, post-CBT FA may result from both IgE-mediated reaction to foods and non-IgE-mediated reaction associated with food protein-induced enterocolitis syndrome (Boyce *et al*, 2010). To our knowledge, this is the first report of post-CBT FA. Patient 1 has been allowed to return to a full diet with the exception of soybeans. As the remaining patients showed no allergic symptoms following the ingestion

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