RESEARCH ARTICLE

Iron refractory iron deficiency anemia: a heterogeneous disease that is not always iron refractory

Albertine E. Donker,[1,2] Charlotte C.M. Schaap,[1,2] Vera M. J. Novotny,[1,3] Roel Smeets,[1,2] Tessa M. A. Peters,[1,2] Bert L. P. van den Heuvel,[1,2] Martine F. Raphael,[4] Anita W. Rijneveld,[5] Inge M. Appel,[6] Andre J. Vlot,[7] A. Birgitta Versluijs,[4] Michel van Gelder,[8] Bernd Granzen,[9] Mirian C.H. Janssen,[10] Alexander J.M. Rennings,[1,10] Frank L. van de Veerdonk,[10] Paul P.T. Brons,[1,11] Dirk L. Bakkeren,[12] Marten R. Nijziel,[1,3,13] L. Thom Vlasveld,[14] and Dorine W. Swinkels[1,2]*

TMPRSS6 variants that affect protein function result in impaired matriptase-2 function and consequently uninhibited hepcidin production, leading to iron refractory iron deficiency anemia (IRIDA). This disease is characterized by microcytic, hypochromic anemia and serum hepcidin values that are inappropriately high for body iron levels. Much is still unknown about its pathophysiology, genotype–phenotype correlation, and optimal clinical management. We describe 14 different *TMPRSS6* variants, of which 9 are novel, in 21 phenotypically affected IRIDA patients from 20 families living in the Netherlands; 16 out of 21 patients were female. In 7 out of 21 cases DNA sequencing and multiplex ligation dependent probe amplification demonstrated only heterozygous *TMPRSS6* variants. The age at presentation, disease severity, and response to iron supplementation were highly variable, even for patients and relatives with similar *TMPRSS6* genotypes. Mono-allelic IRIDA patients had a milder phenotype with respect to hemoglobin and MCV and presented significantly later in life with anemia than bi-allelic patients. Transferrin saturation (TSAT)/hepcidin ratios were lower in IRIDA probands than in healthy relatives. Most patients required parenteral iron. Genotype alone was not predictive for the response to oral iron. We conclude that IRIDA is a genotypically and phenotypically heterogeneous disease. The high proportion of female patients and the discrepancy between phenotypes of probands and relatives with the same genotype, suggest a complex interplay between genetic and acquired factors in the pathogenesis of IRIDA. In the absence of inflammation, the TSAT/hepcidin ratio is a promising diagnostic tool, even after iron supplementation has been given.
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■ Introduction

Matriptase-2, encoded by *TMPRSS6*, plays an essential role in down-regulating hepcidin, the key regulator of iron homeostasis. Pathogenic *TMPRSS6* mutations result in uninhibited hepcidin production, causing iron refractory iron deficiency anemia (IRIDA), a disease characterized by a microcytic, hypochromic anemia due to serum hepcidin values that are inappropriately high for the body iron levels [1–6].

IRIDA patients typically present in childhood with microcytic anemia not responding to oral iron, in combination with remarkably low transferrin saturation (TSAT), which tends to become less severe with increasing age [7]. Serum ferritin levels are generally within the low-normal range, and increase following intravenous (iv) iron treatment [5]. Only a few patients with elevated ferritin concentrations have been described before iv iron treatment had been given [8]. To date, 69 different *TMPRSS6* defects have been identified in 65 IRIDA families with 94 patients of different ethnic origin [4,5,9,10].

At the population level, Genome Wide Association Studies (GWAS) show that *TMPRSS6* is polymorphic with a relatively large amount of polymorphisms of which the nonsynonymous c.2207C>T (p.Ala736Val) is associated with a significant decrease of the concentrations of iron, hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean cellular hemoglobin (MCH), and red blood cells [11,12]. These findings are corroborated by functional studies, which show that the 736Ala variant inhibits hepcidin production more efficiently [13].

TABLE IA. Characteristics of IRIDA Patients. I A. Characteristics of 14 Homozygous or Compound Heterozygous Patients

	Patient characteristics				Laboratory characteristics					Genetic characteristics	Treatment				
			Age years			Hb[a] g/dl	MCV[a] fl	Ferritin[a] µg/l	TSAT[a] %	TSAT/ hepcidin[b] %/nM	TMPRSS6 variant that (probably, possibly) affects function	Oral iron[c]	Iv iron[c]	Other	Duration of treatment	Remarks
ID	Sex	I[d]	II[e]	III[f]						Allele 1;Allele 2					
										deletion; deletion					
1	F	3	9	14	7.9	53	29	4.0	0.27	c.del of 118 kb in 22q12.3[j] c.del of 118 kb in 22q12.3 p.nonsense;p.nonsense deletion; frameshift	+	+	–	Iron iv age 9–14 years	
2[g]	F	2	7	12	6.8	54	53	5.2	0.48	c.del promotor, exon 1–3;c.497delT p.nonsense;p.Leu166Argfs*37 frameshift; frameshift	+	–	BloodTx	BloodTx age 2–8 years	
3	F	4	21	24	7.1	65	44	np	0.59	c.497delT; c.497delT p.Leu166Argfs*37; p.Leu166Argfs*37	+	+	–	Iron iv age 4–21 years	
4[h]	M	child	31	33	10	63	np	2.8	np	c.497delT; c.497delT p.Leu166Argfs*37; p.Leu166Argfs*37	+	+	–	Iron iv as a child	
5	F	5	18	23	8.1	61	16	2.7	1.0	c.1904_1905dup; c.1904_1905dup p.Lys636Alafs*17;p.Lys636Alafs*17	+	+	–	Iron iv at age 19 years	
6	F	18	34	36	7.6	61	18	3.0	np	c.1904_1905dup; c.1904_1905dup p.Lys636Alafs*17;p.Lys636Alafs*17 frameshift; nonsense	+	+	BloodTx	Iron iv from age 34 years	Pregnant at presentation
7	M	0	6	9	5.5	51	np	4.0	0.53	c.497delT; c.1832G>A p.Leu166Argfs*37; p.Trp611X frameshift; missense	+	–	–	Iron po from age <6 years	
8	F	33	38	41	np	np	np	np	0.43	c.497delT; c.1228T>C p. Leu166Argfs*37; p.Cys410Arg	+	–	Iron im	Iron im age 33–38 years	
9	F	27	32	35	7.4	63	66	5.0	0.51	c.497delT; c.1324G>A p.Leu166Argfs*37; p.Gly442Arg	+	+	–	Iron iv age 27–28 years	
10	M	10	12	12	8.4	53	43	2.0	0.13	c.497delT; c.1324G>A p.Leu166Argfs*37; p.Gly442Arg	+	+	–	Iron iv age 10–12 years	Diabetes mellitus
11[i]	F	0	1	2	9.8	66	16	4.0	0.78	c.497delT; c.2383G>A p.Leu166Argfs*37; p.Val795Ile	+	–	–	Iron po from age 1 yr	
12	M	2	3	8	6.4	52	23	4.0	0.53	c.1904_1905dup; c.1229G>C p.Lys636Alafs*17; p.Cys410Ser missense; missense	+	+	–	Iron iv from age 3 years	
13	M	8	8	11	7.9	56	9.0	1.9	0.29	c.521T>C c.1228T>C p.Leu174Pro; p.Cys410Arg	+	–	Vitamin C	Iron po + vitamin C from age 8 years	
14	F	child	44	47	9.3	66	34	3.0	np	c.2383G>A; c.2105G>T p.Val795Ile; p.Cys702Phe	+	+	BloodTx (child)	Iron iv age 36–41 years	Hypothyroidism
Median (range)	M:F 5:9	4.5 (0–33)			7.9 (5.5–10)	61 (51–66)	29 (9–66)	3.5 (1.9–5.2)	0.51 (0.13–1.0)						

TABLE IB. Characteristics of 7 Heterozygous IRIDA Patients

Patient characteristics					Laboratory characteristics					Genetic characteristics	Treatment				Remarks
		Age years								TMPRSS6 variant that (probably, possibly) affects function					
ID	Sex	I[d]	II[e]	III[f]	Hb[a] g/dL	MCV[a] fl	Ferritin[a] µg/L	TSAT[a] %	TSAT/ Hepcidin[b] %/nM	Allele 1;Allele 2	Oral iron[c]	Iv iron[c]	Other	Duration of treatment	
										deletion; Wt[k]					
15[g]	F	10	40	42	5.4	76	np	np	2.7	c.del promotor, exon 1-3; Wt p.nonsense; Wt frameshift; Wt[k]	+	+	—	Start iv iron unknown Iron iv till age of 35 years	
16	F	47	48	53	12	79	130	5.0	0.70	c.497delT; Wt p.Leu166Argfs*37; Wt	+	+	—	Iron iv from age 49 years	
17	F	31	32	33	10	76	32	4.0	0.30	c.497delT; Wt p.Leu166Argfs*37; Wt splicing; Wt[k]	+	+	Vitamin C	Iron iv from age 31 years	
18	F	10	47	48	12	67	np	np	1.1	c.230-6G>A[l]; Wt splicing; Wt	+	+	—	Iron iv when a child No treatment anymore	
19	F	39	41	46	10	78	53	6.0	1.0	c.863+1G>T[m]; Wt splicing; Wt missense; Wt[k]	+	—	—	Iron po from age 31 years	
20	F	43	43	44	7.9	62	10	2.7	np	c.1654G>A; Wt p.Asp552Asn; Wt	+	+	BloodTx	Iron iv from age 40 years	
21	F	31	34	40	9.7	68	22	4.0	2.6	c.2105G>T; Wt p.Cys702Phe; Wt	+	—	—	Iron po from age 31 years	
Median (range)	M:F 0:7	31 (10–47)			10 (5.4–12)	76 (62–79)	32 (10–130)	4.0 (2.7–6.0)	1.1 (0.33–2.7)						

Patients are ordered according to type of variant. Family studies have confirmed that variants of all compound heterozygous affected patients are located on two different alleles.

Hb = hemoglobin; MCV = mean corpuscular volume; TSAT = transferrin saturation; F = female; M = male; iv = intravenous; po = per os; im = intramuscular; BloodTx = blood transfusion; np = not provided; Wt = wild-type.

a Hb, MCV, ferritin, and TSAT of the proband at time of presentation with anemia.

b Hepcidin, TSAT/hepcidin ratio at time of (genotypic) diagnosis of IRIDA, in absence of inflammation except for patient 14 and 20 with CRP levels of 24 and 45 mg/l, respectively. The median reference level of serum hepcidin-25 is 4.5 nM for men (p 2.5–p 97.5 $\leq$ 0.5–14.7 nM), 2.0 nM for premenopausal women (p 2.5–p.97.5 = 0.1–12.3 nM), and 4.9 nM for postmenopausal women (p 2.5–p 97.5 = 0.2–15.6 nM). The reference level of serum hepcidin-25 for children aged 0.5 – 3 years is 3.6 nM (p 2.5–p.97.5 = 0.94–12.2). For children >3 years no reference ranges are available at present, so we recommend for them using those of premenopausal women. The median reference level of serum TSAT/hepcidin-25 ratio is 7.3%/nM for men (p 2.5–p 97.5 = 1.7–256.3%/nM), 13.9%/nM for premenopausal women (p 2.5–p 97.5 = 2.0–330%/nM) and 5.7%/nM for postmenopausal women (p 2.5–p97.5 = 1.5–73.4%/nM) (http://www.hepcidinanalysis.com) [23].

c +: has been given, –: has not been given.

d I, age at presentation with anemia.

e II, age at assessing IRIDA and determination of hepcidin and TSAT/hepcidin ratio.

f III, age in 2015.

g Patient 2 is the daughter of patient 15. However, both patients presented with anemia at childhood, both were considered as independent probands.

h Patient 4 was diagnosed with anemia refractory to oral iron as a child for which he temporarily received intravenous iron. IRIDA was diagnosed after screening for this disorder because of pregnancy of his wife. No data are available on Hb and iron parameters at time of presentation of anemia.

i Hb and iron parameters of patient 11 at referral, after oral iron supplementation had been given.

j Deletion 118 kb in intron 2, knocking out exon 3–18 of TMPRSS6 gene. Also other genes were deleted; RefSeq genes TEX33, MPST, TST, and KCTD17.

k Multiplex ligation dependent probe amplification (MLPA) confirmed heterozygosity.

l This substitution is predicted to introduce a new and more efficient acceptor splice site 4 bases downstream from the original acceptor splice site in intron 2 leading to a frameshift in the open reading frame. Not proven on RNA or protein level (http://www.interactive-biosoftware.com/doc/alamut-visual/2.7/splicing.html), accessed July 14, 2016.

m This substitution is located in the donor splice site of intron 7. The consequence of this change is not predictable, but a skip of exon 7 is very likely resulting in the loss of a classical splice site. Not proven on mRNA or protein level (http://www.interactive-biosoftware.com/doc/alamut-visual/2.7/splicing.html), accessed July, 14 2016.

The increasing number of IRIDA cases that are being reported, generate more knowledge on the disease, but many questions remain concerning the mode of inheritance, the genotype–phenotype correlation, the diagnostic workup, and the optimal treatment.

Since usually bi-allelic *TMPRSS6* variants are found in IRIDA patients, the disease is considered as recessive. However, anecdotal data are available of phenotypically affected IRIDA patients in which only a heterozygous *TMPRSS6* variant was found [3,14–17].

Concerning the genotype–phenotype relationship, there is a tendency towards lower Hb, MCV and TSAT in patients with two nonsense mutations [10]. Much is unknown on the influence of high frequency *TMPRSS6* variants, other still unrecognized genes and environmental factors in the phenotypic expression of the disease. Furthermore, diagnosing IRIDA is challenging because of the highly variable phenotype [4,5,10] and the unclear genotype–phenotype relation. The optimal oral and/or iv iron treatment regimen has not been established yet.

In this paper, we describe the characteristics of 21 IRIDA patients and relatives in the Netherlands, the relation between genotype and phenotype in terms of age of presentation, severity of anemia, and response to iron supplementation. Our observations add to the understanding of the clinical and genetic heterogeneity of IRIDA. They moreover suggest the TSAT/hepcidin ratio as a promising tool in the diagnosis of IRIDA.

■ Methods

Patients and relatives. We included 21 IRIDA patients and their relatives. All IRIDA patients were inhabitants of the Netherlands and consecutively diagnosed between 2010 and 2015.

IRIDA probands were defined as patients with both an "IRIDA phenotype" (detected after clinical presentation, microcytic anemia, TSAT < 10%, in the absence of inflammation, Hb and MCV not or partially responsive to oral iron) and an "IRIDA genotype" (a mono- or bi-allelic *TMPRSS6* variant that—probably, possibly—affects function, called "defect" hereafter). Iron deficiency anemia in these patients could not be explained (exclusively) by increased physiological needs (growth, menstrual blood loss in premenopausal women), by gastrointestinal disease like *Helicobacter pylori* infection, celiac disease or atrophic gastritis [18] or by gynecological problems. Responsiveness to oral iron treatment was defined as a hemoglobin (Hb) increment of 2 g/dL after 3 weeks of oral iron supplementation [19]. A positive family history for unexplained iron deficiency anemia and anemia presenting in childhood were considered as suspect for IRIDA.

Laboratory measurements. Hb, red blood cell indices, and serum iron parameters were measured in accredited Dutch hospital laboratories. Serum hepcidin measurements were performed by a combination of weak cation exchange and time-of-flight mass spectrometry (WCX-TOF MS) [20,21]. The median reference level of serum hepcidin-25 is 4.5 nM for men (p 2.5–p 97.5 ≤ 0.5–14.7 nM), 2.0 nM for premenopausal women (p 2.5–p 97.5 = 0.1–12.3 nM) and 4.9 nM for postmenopausal women (p 2.5–p 97.5 = 0.2–15.6 nM). The reference level of serum hepcidin-25 for children aged 0.5–3 years is 3.6 nM (p 2.5–p.97.5 = 0.94–12.2) [22,23]. For children >3 years no reference ranges are available until now, so for this group we recommend using those of premenopausal women. The median reference level of serum TSAT/hepcidin-25 ratio is 7.3%/nM for men (p 2.5–p 97.5 = 1.7–256.3%/nM), 13.9%/nM for premenopausal women (p 2.5–p 97.5 = 2–330%/nM) and 5.7%/nM for postmenopausal women (p 2.5–p97.5 = 1.5–73.4%/nM) [23].

Gene analysis. Genotyping was performed by PCR, DNA Sanger sequencing (until March 2014) and Ion Torrent sequencing (after March 2014) of the coding part of *TMPRSS6* (Supporting Information Table 1). Comparative genomic hybridization as performed in patient 1 and her relatives, for whom PCR products of the *TMPRSS6* gene could not be obtained, using the Affymetrix CytoScan HD array platform according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA). Multiplex ligation-dependent probe amplification (MLPA) analysis was performed in patients who had an IRIDA phenotype and a mono-allelic pathogenic *TMPRSS6* variant to exclude large deletions and/or duplications in the 'healthy' allele, and also in patient 1 (Supporting Information Table 2, The Netherlands, www.mlpa.com) [24].

The pathogenicity of genetic variants was assessed by review of the literature on previous reported cases and functional studies, association of the variant with the phenotype within a family and bioinformatic tools (SIFT, Align GVGD, Polyphen, SpliceSiteFinder-like, MaxEntScan, NNSplice, GeneSplicer, and Human Splicing Find, all as part of Alamut software, Alamut) [25]. Alamut was used to assess pathogenicity in case of not previously reported *TMPRSS6* variants. Haplotype analysis was performed in a search for a founder effect in families with identical pathogenic *TMPRSS6* defects using 10 different intragene exon high frequency variants (HFVs)
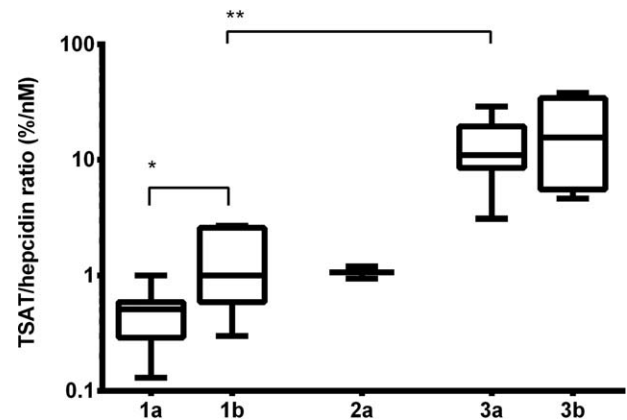

Figure 1. TSAT/hepcidin ratio in bi-allelic (1a) and mono-allelic (1b) affected IRIDA patients and their clinically not affected relatives (2a, 3a, 3b). Patients are defined as having both an IRIDA phenotype (detected after clinical presentation, microcytic anemia, TSAT below the reference range, in the absence of inflammation, not or partially responsive to oral iron) and an IRIDA genotype (a mono- or bi-allelic pathogenic defect in the *TMPRSS6* gene). 1. Patients with an IRIDA phenotype; 1a. Probands with bi-allelic *TMPRSS6* defect, *n* = 11; 1b. Probands with mono-allelic *TMPRSS6* defect, *n* = 6, and affected relative (mother of patient 17) with mono-allelic *TMPRSS6* defect, *n* = 1; 2. Relatives without an IRIDA phenotype. 2a. Relatives with bi-allelic *TMPRSS6* defect, *n* = 2; 3. Relatives without an IRIDA phenotype; 3a. Relatives with mono-allelic *TMPRSS6* defect, *n* = 14; 3b. Wild-type *TMPRSS6* relatives, *n* = 4 Patients and relatives with signs of inflammation were excluded from the analysis. Boxes indicate median and interquartile ranges; whiskers describe the range of the data (min–max). *P < 0.05; **P < 0.001 as tested by unpaired *t* test.

detected by Sanger DNA sequencing and 3 short tandem repeats surrounding *TMPRSS6*. We also performed parental haplotype analysis in two sibling pairs with identical heterozygous *TMPRSS6* variants but different phenotypes, using seven different intragene HFV's also detected by Sanger DNA sequencing.

■ Results

Patient characteristics

We report 21 IRIDA patients from 20 unrelated families. IRIDA was diagnosed in case of the combination of a relevant phenotype and genotype.

After exclusion of other conditions as the (exclusive) explanation, a patient with a current or past microcytic hypochromic anemia, in absence of inflammation, not or partially responsive to oral iron with a low TSAT (<10%) was considered as a suspected case, after which serum hepcidin analysis was performed in 17 out of 21 patients and genotyping of *TMPRSS6* in all patients.

In our diagnostic work-up, very low to elevated ferritin levels, either before or after treatment with iron supplementation could fit the diagnosis.

Nine female and five male patients were diagnosed with a bi-allelic *TMPRSS6* defect (Table IA). In patient 1, comparative genomic hybridization was performed since no signal of *TMPRSS6* was obtained by Sanger sequencing. A homozygous deletion of 118 kb was detected in 22q12.3, with the distal breaking point in intron 2 of the *TMPRSS6* gene (deletion breakpoints at Mb positions 37,374,751 and 37,492,851), effectively knocking out exons 3–18 (UCSC Genome Browser on Human Feb 2009 GRCh 37/hg 19 assembly [26]; Supporting Information Data Fig. 1). In the other 7 patients (all female) only a mono-allelic *TMPRSS6* defect was found (Table IB). In these patients MLPA showed no large deletions and/or duplications in the 2nd allele.

For the bi-allelic affected patients, age at the time of evaluation for IRIDA ranged from 1 to 40 years; 10 out of 14 patients were in their first or second decade. Evaluation for IRIDA of the seven heterozygous patients all occurred in the third or fourth decade.

TABLE II. Characteristics of Novel *TMPRSS6* Variants

	TMPRSS6 defect[a]					
Gene	Protein	Type of variant	TMPRSS6 domain	*In silico* findings	Conclusion on pathogenicity	Patient ID
c.del 118 kb intron 2–exon 3–18	Absent	Deletion	Absent protein	np	Pathogenic	1
c.del promotor, exon 1–3	Absent	Deletion	Absent protein	np	Pathogenic	2, 15
c.230-6G>A	Aberrant	Splicing defect	TM domain	Splice site[b]	Most probably pathogenic	18
c.521T>C	p.Leu174Pro	Substitution	SEA domain	Align GVGD[c]: C0 Polyphen[d]: 0.57 SIFT[e]: 0.04	Possibly pathogenic	13
c.863 + 1G>T	del exon 7	Splicing defect	CUB domain	Skip of exon 7[f]	Most probably pathogenic	19
c.1228T>C	p.Cys410Arg	Substitution	CUB domain	Align GVGD[c]: C0 Polyphen[d]: 0.45 SIFT[e]: 0.00	Possibly pathogenic	13
c.1229G>C	p.Cys410Ser	Substitution	CUB2 domain	Align GVGD[c]: C0 Polyphen[d]: 0.01 SIFT[e]: 0.00	Possibly pathogenic	12
c.1654G>A	p.Asp552Asn	Substitution	LDLR domain	Align GVGD[c]: C0 Polyphen[d]: 1.0 SIFT[e]: 0.00	Possibly pathogenic	20
c.1832G>A	p.Trp611X	Stop codon	Serine Protease domain	np	Most probably Pathogenic[g]	7

np = not provided.

[a] All novel *TMRPSS6* variants, except the defect c.del promotor, exon 1–3 in patient 15, were found in combination with a *TMPRSS6* variant that (probably, possibly) affects function, on the other allele. Therefore, the contribution of the novel variant to the clinical phenotype of the patient remains unclear.

[b] This substitution is predicted to introduce a new and more efficient acceptor splice site 4 bases downstream from the original acceptor splice site in intron 2 leading to a frameshift in the open reading frame. Not proven on RNA or protein level (http://www.interactive-biosoftware.com/doc/alamut-visual/2.7/splicing.html, accessed July 14, 2016).

[c] **Align GVGD,** web based *in silico* prediction software program that combines the biophysical characteristics of amino acids and protein multiple sequence alignments to predict where missense substitutions in genes of interest fall in a spectrum from enriched deleterious to enriched neutral. A-GVGD scores amino acid substitutions on a 7-scale scoring system, from C0 to C65. An amino acid substitution with a C0 score is considered to be neutral, amino acids with C15 and C25 scores are considered intermediate, as changes to protein structure or function are uncertain, and C35 scores or higher are considered as likely deleterious [52].

[d] **PolyPhen-2** (Polymorphism Phenotyping v2 HumVar) is a tool which predicts possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations. PolyPhen scores range from $0 \leq 1 \leq X$, outcome scores of 0.00–0.15 are classified as benign, 0.15–1.0 as possibly damaging, 0.85–1.0 as more confidently predicted to be damaging [53].

[e] **The SIFT** algorithm combines sequence homology and physical properties of amino acid substitutions to analyze whether or not amino acid substitutions are tolerated, in light of the predicted effect on the protein structure. SIFT score ranges from 0 to 1. The amino acid substitution is predicted damaging if the score is <0.05 and tolerated if the score is >0.5 [54].

[f] This substitution is located in the donor splice site of intron 7. The consequence of this change is not predictable, but a skip of exon 7 is very likely as *in silico* analysis resulting in the loss of a classical splice site. Not proven on mRNA or protein level (http://www.interactive-biosoftware.com/doc/alamut-visual/2.7/splicing.html, accessed July 14, 2016).

[g] Nonsense substitution in exon 15. The reading frame is interrupted by a stop codon. The mRNA produced might be targeted to nonsense-mediated decay (NMD). No formal proof.

All 14 patients diagnosed with a bi-allelic *TMPRSS6* defect had been treated with oral iron at referral to our clinic. Of these patients two were responsive to oral iron with a slow, sluggish increase of Hb and MCV (ID 7, ID 11), while patient 13 responded to a combination of oral iron and vitamin C with an increase of Hb, MCV, and TSAT. The other 11 bi-allelic patients were unresponsive to oral iron and partially responsive to parenteral iron or blood transfusions in terms of a moderate increase of Hb and MCV, while TSAT remained below 10% in 9 out of 11 patients (ID 8: TSAT 13%, ID 14: TSAT 15% after iron treatment) (Table IA and Supporting Information Table 3).

All seven patients diagnosed with a heterozygous *TMPRSS6* defect had been treated with oral iron at referral to our clinic, of which two were responsive (ID 19 and 21) with a slow, sluggish increase of Hb, MCV, and TSAT. The other five patients received iv iron supplementation, which resulted in a moderate increase of Hb and MCV. TSAT remained below reference values, except in patient 15. One heterozygous patient (ID 20) received a blood transfusion. Ferritin increased above concentrations of 500 μg/L in 3 out of 21 patients (patient 8, 14, 16).

In patient 8 (ferritin 924 μg/l) MRI of the liver showed only moderate signs of iron overload [60 μmol (or 3.35 mg) Fe per gram dry weight tissue]. Since no genotyping of the known hemochromatosis genes was performed for our patients, we cannot exclude ferroportin disease due to a variant of *SLC40A1* for this woman.

Dose of iv iron, treatment regimen and duration of treatment was highly variable in our small population, with a tendency to a reduction of iv iron requirements with increasing age. None of our patients was treated with erythropoietin (EPO). We conclude that only a minority of both bi-allelic and mono-allelic affected patients was responsive to oral iron and that most patients needed parenteral iron to increase their Hb levels.

Genotypic and phenotypic family screening was performed in 27 relatives of 12 out of 21 probands; in 20 out of these 27 relatives a TSAT/hepcidin ratio was available. Four wild-type relatives and 20 relatives with a mono-allelic *TMPRSS6* defect had no IRIDA phenotype. Two relatives with a bi-allelic *TMPRSS6* defect (brother of patient 9 and sister of patient 11) had no complaints since their Hb was normal (MCV and TSAT were decreased). Only the mother of patient 17, who had the same heterozygous genotype as her daughter, had an IRIDA phenotype. She had a history of unexplained iron deficiency (Supporting Information Table 4).

Molecular characterization

We observed 14 different defects in 21 patients (Table IA, Supporting Information Table 5). Five out of 14 are previously described *TMPRSS6* defects: 3 missense variants [3,15,27], and 2 frameshift variants [3,15]. Of these variants, the frameshift alteration c.497delT (p.Leu166Argfs*37) was the most prevalent defect in our population and was found in 8 out of 14 bi-allelic patients and in 2 out of 8 mono-allelic patients (12 out of 36 affected alleles = 33%) (Table IA). All patients with the c.497delT (p.Leu166Argfs*37) were of Dutch origin, whereas all three patients with the c.1904_1905dup (p.Lys636Alafs*17) were of Turkish origin (Supporting Information Table 6).

For the nonsynonymous missense variant c.2383G>A (p.Val795Ile) a functional test had previously been performed. This showed physiological inhibition of *HAMP1* expression (*in vitro* studies by site directed mutagenesis into murine *TMPRSS6* [15]), despite the IRIDA phenotype of the patient who harbored this variant. However, considered as the most reliable method to predict the pathogenicity of an allele variant, the results of cell based functional testing by site directed mutagenesis should be interpreted with caution. The technique has been described as error prone and is likely to be limited in its extrapolation because the test environment may not fully reflect the *in vivo* situation [15,28]. Allele frequency of the c.2383G>A (p.Val795Ile) missense variant is <1% (Supporting Information Table 5), which argues against a neutral or beneficial effect of this nucleotides change. Because of the not fully reliable functional tests, the low allele frequency and the *in silico* analysis that point towards pathogenicity, we considered this missense variant as pathogenic [29].

Nine novel pathogenic *TMPRSS6* defects were demonstrated in our case series; 2 large deletions, 4 missense mutations, 1 nonsense mutation, and 2 splice defects (Table II). Pathogenicity of the new mutations was predicted with *in silico* software. In all seven heterozygous IRIDA patients, no deletions and/or duplications in the *TMPRSS6* gene of the 'healthy' allele were found with MLPA.

Haplotype analysis exploiting 10 intragenic HFVs with a minor allele frequency in Caucasians of >0.005 and 3 short tandem repeats surrounding *TMPRSS6* was performed in patients with identical defects. The results were consistent with a common ancestor of the Dutch patients 3 and 4, who all carried the c.497delT defect. For the other patients with this defect the haplotype analysis might indicate relationship, but was less clear. The haplotype analysis of patients with the c.1904_1905dup defect, found in three patients (5, 6, and 12) from Turkish origin, was consistent with a common ancestor of the patients 5 and 6. Patients 8 and 13 who carried the c.12282T>C defect might be related but haplotype analysis was not clearly indicative (Supporting Information Table 7).

We conclude that (i) in the Netherlands certain defects are more prevalent than others, (ii) patients who share a country of origin are likely to share specific defects, and (iii) common ancestry of patients with identical defects is not always obvious.

Genotype–phenotype relation

The IRIDA phenotype was highly variable in our population (Table IA). To improve insight in genotype–phenotype relation we categorized the probands by six different groups based on the nature of the defect (Table IA). Patients with large deletions either bi-allelic (ID 1) or in combination with a frameshift defect (ID 2) had the most severe IRIDA phenotype with respect to age of presentation, severity of anemia, microcytosis, and duration of treatment with iv iron. However, patient 3 and 4, who shared the same homozygous frameshift defect, had very different phenotypes; patient 4 was only temporarily treated with iv iron as a child while patient 3 received iv iron till the age of 21 years. Only 2 of the 14 bi-allelic patients

responded to oral iron supplementation, i.e., patient 7 and 11. Interestingly, these patients had severe genotypes. Patient 13 with two missense defects was responsive to a combination of oral iron with vitamin C.

Seven patients were diagnosed with IRIDA due to a variety of heterozygous *TMPRSS6* defects (Table 1B). The large deletion in patient 15 resulted in a more severe genotype than the frameshift, aberrant splicing and missense defects in the other heterozygous patients.

Overall (i) patients with bi-allelic, severe gene defects had the most severe phenotype, but exceptions occurred and genotype alone was not fully predictive for the response to oral iron, (ii) mono-allelic patients presented later in life with anemia (median 31 years, range 10–47 years) than bi-allelic patients (median 4.5 years, 0–33 years, Mann–Whitney $P = 0.03$), were more likely to be females and had a milder phenotype with respect to severity of anemia, microcytosis, and duration of iv iron treatment.

Since patients with identical defects were scarce, our patient series do not allow to decipher a clear influence of the presence of the high frequency *TMPRSS6* variant c.2207C>T (p.Ala736Val) on the severity of the disease (Supporting Information Table 6). Nevertheless, our data suggest that for prediction of the phenotype, the nature of the pathogenic *TMPRSS6* defect overrides a possible influence of the c.2207C>T (p.Ala736Val) variant.

Interestingly, mono-allelic mutated relatives with the same *TMPRSS6* defect as the probands, screened at the time of diagnosis of the proband, were not affected at all, except for the mother of patient 17.

In two sibling pairs with identical heterozygous *TMPRSS6* variants but different phenotypes parental haplotype analysis was performed (Supporting Information Table 8). In the family of patient 19, both the female proband and her unaffected sister inherited the mutant allele from their mother. However, they received different paternal alleles. Their unaffected brother received two wild type alleles from his mother and father. In the family of patient 20, genetic data were only available from the proband and her sister, not from the parents. Both the female proband and her unaffected sister shared the same *TMPRSS6* variant, but had different wild type alleles, indicating different parental haplotypes of the 'healthy' allele. Although based on analysis of only two families these data suggest that in individuals with a heterozygous *TMPRSS6* variant, differences in their second allele that is designated as "wild type" account for difference in IRIDA phenotype.

Diagnostic value of the TSAT/hepcidin ratio and the oral iron absorption test

TSAT/hepcidin ratio in IRIDA patients and their unaffected relatives. We evaluated TSAT/hepcidin ratios in (i) bi-allelic probands, (ii) mono-allelic probands, (iii) bi-allelic relatives, (iv) mono-allelic relatives, and (iv) wild-type relatives for whom the TSAT and hepcidin were determined and who had no signs of inflammation. TSAT/hepcidin was significantly lower for bi-allelic IRIDA patients (median: 0.51%/nM, range 0.13–1.0%/nM, $n = 11$) than for mono-allelic IRIDA patients (1.0%/nM, 0.3–2.7%/nM, $n = 7$, unpaired *t* test $P < 0.05$). Interestingly, mono-allelic IRIDA patients had lower ratios than their relatives with the same genotype but without an IRIDA phenotype (11%/nM, 3.1–29%/nM, $n = 14$, $P < 0.001$). Among relatives without a phenotype, the ratios were similar for mono-allelic (11%/nM, 3.1–29%/nM, $n = 14$) and wild-type subjects (16%/nM, 4.6–38%/nM, $n = 4$) (Fig. 1).

Iron oral absorption test in the diagnosis of IRIDA patients. To evaluate intestinal iron absorption, an iron oral absorption test (IOAT) was performed in 2 bi-allelic and 3 mono-allelic IRIDA patients [30,31]. In the bi-allelic patients no enteral iron absorption

was demonstrated. In the mono-allelic patients, the quantitative results of the IOAT did not predict the severity of the phenotype and the response to oral iron (Supporting Information).

Ferrokinetic studies. Prior to diagnosis of IRIDA, ferrokinetic studies with radioactive labeled iron were performed in patients 3, 14, and 15 in order to evaluate mucosal iron uptake, transfer, plasma iron clearance, red cell iron incorporation, and iron retention [32–34]. The data showed a defect in intestinal iron uptake but adequate iron uptake and incorporation by erythroblasts illustrating that IRIDA is a defect of cellular iron release and that erythroblast iron uptake from the circulation and incorporation occurs fast and without restrictions (Supporting Information Table 9).

The kinetics of the effect of iv iron on blood and serum parameters was studied by administration of 200 mg iv iron in patient 14, followed by measurements during the first day and at $t = 1$ week. Results demonstrated a significant but temporary increase of serum hepcidin on day 1 and a slight increase of Hb, MCV, and ferritin after 1 week. TSAT and the TSAT/hepcidin ratio remained low (Supporting Information Table 10).

■ Discussion

We describe a Dutch case series of 21 IRIDA patients and their relatives. We found that patients with bi-allelic severe genotype defects had more severe IRIDA phenotypes than patients with milder *TMPRSS6* defects. This corresponds to scarce data in literature [4,5] where a tendency to a more severe IRIDA phenotype has been described in patients with bi-allelic nonsense *TMPRSS6* defects compared to patients with missense defects. However, our data show that exceptions occur.

We observed that for as many as 7 out of 21 of our patients only a heterozygous *TMPRSS6* defect was found. MLPA showed no deletions or duplications in the 'healthy' allele. We appreciate that we did not exclude mutations and deletions in the promotor region, deep intronic inversions or balanced translocations of chromosome 22. However, our findings corroborate previous reports on mono- allelic patients that present with an IRIDA phenotype, which is generally mild (reviewed in ref. [4]). Interestingly, none of the relatives with a heterozygous *TMPRSS6* defect—except for one—were affected. We hypothesize that differences in phenotype between probands and relatives with identical genotypes might be ascribed to a different expression of the wild type *TMPRSS6* allele compared to the affected allele in patients and in unaffected individuals [35]. Indeed, Serre et al describe an array-based analysis of 643 genes expressed in lymphoblastoid cell lines, which shows that for a large proportion (22%) of them, including *TMPRSS6,* the two alleles are differentially expressed. This imbalance in allelic expression can at least partially be explained by epigenetic mechanisms such as lyonization in females and imprinting [36]. This hypothesis is substantiated by the results of our parental haplotype analysis performed in two families suggesting that differences in phenotype between probands and unaffected siblings with the same heterozygous *TMPRSS6* defect is attributable to differences in inherited parental wild type alleles. The finding that the TSAT/hepcidin ratio is lower for bi-allelically mutated probands, than for mono-allelically mutated probands, reflects that the degree of dysregulation of hepcidin production in IRIDA probands is a sliding scale that correlates with the severity of the genotype. These observations suggest that the mode of inheritance in IRIDA is complex. Overall, our small amount of data on the genotype-phenotype correlation in IRIDA patients and their relatives support the notion that phenotypical penetrance of *TMPRSS6* defects is influenced by other (epi)genetic and environmental factors such as growth, co-morbidity as inflammation and blood loss, corroborating some previous observations in mice and man [37].

Up to now, 94 IRIDA patients of different ethnic origin with 69 different homozygous or compound heterozygous *TMPRSS6* defects have been described in 65 families [4,5,9,10]. Our case series of 21 patients adds 9 new mutations, spread throughout the entire matriptase-2 protein and illustrates a geographical distribution of *TMPRSS6* defects with different mutations in the patients from Dutch versus Turkish descent. However, haplotype analysis could not prove common ancestry of all patients with identical defects.

Since the cardinal feature of IRIDA is a discrepantly high serum hepcidin in relation to the low iron body status, we hypothesized that the TSAT/hepcidin ratio as first mentioned by Heeney [38] could be a useful diagnostic tool. In our population, consisting of clinically presenting patients and their relatives, TSAT/hepcidin ratio was able to discriminate between bi-allelic and mono-allelic IRIDA patients, and between mono-allelic IRIDA patients and their phenotypically unaffected relatives with the same heterozygous *TMPRSS6* defect, even after iron supplementation had been given, provided that inflammation was absent. However, before its introduction as a diagnostic test in the work up of iron deficient microcytic anemic patients suspected for the presence of IRIDA, the ratio needs confirmation in phenotypically and genotypically proven IRIDA patients versus patients presenting with an iron deficient microcytic anemia because of other reasons, e.g., inadequate intake, blood loss or other forms of refractory IDA, such as celiac disease, autoimmune gastritis, and *Helicobacter pylori*.

In five of our patients an OIAT test was performed [30,31]. Results were abnormal but since quantitative results did not correlate with the severity of the phenotype and response to oral iron therapy, we concluded that the diagnostic value of this test is limited in the work up of patients suspected or diagnosed with IRIDA.

As the acronym IRIDA implies, patients with *TMPRSS6* defects are usually unresponsive to oral iron. However, as shown by our case series and also by others in some IRIDA patients with both mono-allelic and bi-allelic *TMPRSS6* defects it is possible to increase the Hb to a clinically acceptable level with only oral iron, or with a combination of oral iron and vitamin C [39]. Nevertheless, most IRIDA patients require parenteral iron in order to correct the anemia. According to the literature and to our data, there is a tendency to a reduction of iv iron requirements with increasing age. To our knowledge, no studies are available on the optimal dose and dosing intervals of iv iron in IRIDA patients. Kitsaki et al [40] investigated hepcidin and other iron parameters in hemodialysis patients, characterized by elevated hepcidin levels [41] treated with iv iron. They observed a rapid, small but significant elevation and subsequent decrease to baseline level of serum hepcidin after administration of iv iron, as also seen in patient 14 (Supporting Information Table 9). This increase of hepcidin inhibits the efflux of iron from the macrophages to the serum, thereby decreasing the amount of iron supply to transferrin and erythroblasts. This suggests that small doses of iv iron with short intervals resulting in only slightly elevated hepcidin levels might be superior to large doses with large intervals with regards to increasing the serum levels of ferric-transferrin available for erythropoiesis and minimizing substantial iron sequestration of the reticulo-endothelial system (RES) (and associated elevation of ferritin levels) [40]. These observations need confirmation in IRIDA patients.

Since IRIDA is a disease with inappropriately high serum hepcidin levels and a low TSAT, excess of iron due to iv iron treatment will be stored in the RES of especially the liver and the spleen. This predominantly RES iron storage is also characteristic for patients with loss of function variants in *SLC40A1,* but differs from observations in patients with hereditary hemochromatosis due to variants in *HFE, TfR2, HJV* and *HAMP* and gain of function variants in *SLC40A1* that is associated with iron accumulation in the parenchymal cells, such as

the hepatocytes, which can be harmful and of more concern than iron stored in the RES [42–44]. However, long-term consequences of iv iron treatment on for instance the occurrence of infections and tissue damage have not been established yet in IRIDA patients. Therefore, to stay on the safe side and based on the case report of Cau [39] and on the clinical course of patient 13, we recommend performing a trial of oral iron in combination with vitamin C in IRIDA patients not responsive to oral iron alone, prior to starting parenteral iron supplementation, in order to use the more natural intestinal function of the intestine to prevent toxic iron loading. In addition, since the clinical phenotype of an IRIDA patient might evolve in time from unresponsiveness to partially responsiveness to oral iron [45,46], we suggest to critically assess whether repeated administration of iv iron is required, and to reassess responsiveness to oral iron and vitamin C.

Five out of our 21 patients have a Mediterranean origin. In addition, 40 out of 61 bi-allelic and 6 out of 10 mono-allelic patients described to date are from thalassemia or malaria endemic regions [3,4,7]. It is known that especially β-thalassemia, but also severe α-thalassemia (HbH disease) patients have relatively low serum hepcidin levels due to an ineffective erythropoiesis [47,48]. In β-thalassemia mice, TMRPSS6 defects counteract the low serum hepcidin levels, thereby attenuating iron overload and anemia [37,49,50]. Alternatively, since Plasmodium falciparum has been reported to infect iron-deficient erythrocytes less efficiently, TMPRSS6 defects may also directly protect against malaria [51]. Altogether, these data suggest a survival benefit for patients with malaria or thalassemia syndromes that also harbor a TMPRSS6 defect.

Taken together, in the present study we further substantiate previous observations that (i) mono-allelic TMPRSS6 defect may result in a clinical phenotype of IRIDA that is generally milder than in patients with a bi-allelic TMPRSS6 defect, (ii) IRIDA is a defect of cellular iron release as shown by ferrokinetic studies, (iii) despite the acronym IRIDA, some patients benefit from treatment with oral iron, (iv) IRIDA due to (a) TMPRSS6 defect(s) is a phenotypically and genotypically heterogeneous disease.

Our novel observations include: (i) identification of nine not previously described TMPRSS6 defects, (ii) the presence of country/region specific TMPRSS6 defects, (iii) a relatively high number of patients (all females) were only mono-allelically affected, and (iv) in the absence of inflammation, a low TSAT/hepcidin ratio was associated with TMPRSS6 defects and an IRIDA phenotype, even after iron supplementation has been given. Implications of our findings for clinical management of IRIDA patients may comprise: (i) the TSAT/hepcidin ratio may prove to be a suitable parameter to detect IRIDA patients among patients presenting with unexplained microcytic anemia with a low TSAT and (ii) oral iron with vitamin C treatments should be (re)considered prior to starting iv iron.

In time, suppression of the hepcidin pathway may become an alternative therapeutic approach, especially for severely affected patients to prevent iron overload, but to date safety and benefits remain unknown, especially for pediatric patients. Further studies are required to (i) assess the value of the TSAT/hepcidin ratio in the differential diagnosis of microcytic anemia, (ii) elucidate the contribution of other (epi)genetic and environmental factors in the pathophysiology and clinical penetrance of TMPRSS6 defects, and to (iii) determine the optimal treatment regimen. However, in order to prevent misdiagnosis and unnecessary invasive diagnostic work up, the first challenge for the clinician remains the recognition of the disorder and differentiation from other common causes of microcytic anemia.

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■ Author Contributions

Contribution: A.E.D is a pediatric hematologist and PhD candidate who collected data, analyzed results, reviewed the literature and drafted the manuscript, with help of C.C.S and T.M.A.P V.M.J.N, M.F.R, A.W.R, I.M.A, A.J.V, A.B.V, M.G, B.G, M.C.H.J, A.J.M.R, F.L.V, M.R.N, L.T.V, P.P.T.B are clinicians who diagnosed and treated the patients and provided clinical data for this paper. R.S and B.L.P. H are geneticists who coordinated the genotyping of patients and drafted the methods of the genetic part of the paper. D.L.B is a clinical chemist who provided data for this study. D.W.S initiated and coordinated the project. All authors approved the final version of the manuscript.

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