The role of Matriptase-2 during the early postnatal development in humans

The hepatic hormone hepcidin is a key regulator of systemic iron homeostasis. It limits both iron absorption from the intestine and iron release from macrophage stores by binding to ferroportin and triggering its internalization and degradation. Hepcidin expression is modulated in response to several physiologic and pathologic stimuli, which include systemic iron loading, erythropoietic activity and inflammation.[1] A type II transmembrane serine protease matriptase-2 (MT-2, encoded by TMPRSS6) was identified as a repressor of hepcidin expression acting through the interruption of BMP6/HJV/SMAD signaling.[2-4] Thus, by downregulating hepcidin gene expression, MT-2 controls iron availability to avoid systemic iron deficiency.[5,6]

Mutations in *TMPRSS6* result in clinical phenotype of iron refractory iron deficiency anemia (IRIDA)[7] characterized by hypochromic microcytic anemia, low transferrin saturation and inappropriate normal/high levels of hepcidin. Up to now 69 different mutations in the *TMPRSS6* gene have been reported in 65 IRIDA families with patients of different ethnic origin.[8,9]

Even considering all of the cases published so far, experience with the natural history of IRIDA patients is limited, and data are not reported for the neonatal period. Thus, from the clinical histories, it remains unknown whether IRIDA patients are already iron-deficient at birth.

The aim of this study is to clarify whether matriptase-2 has a role in human fetuses/neonates for a better understanding of iron homeostasis during early development.

Four families (with seven probands) were collected whose pedigrees are shown in Figure 1. Results on ethnic origin, clinical, genetic and laboratory tests are shown in *Online Supplementary Table S1*. These studies were approved by the Institutional Review Board of the Federico II University Medical School in Naples, and conducted in accordance with the Declaration of Helsinki.

The conditions of DNA extraction, polymerase chain reaction, and sequence analysis used were standard. All exons, exon–intron boundaries and a varying amount of the 5' and 3' flanking sequence of the *TMPRSS6* gene were examined using fluorescent chain-terminator cycle sequencing. Detailed protocols and primer used for sequencing sequences are available in *Online Supplementary Methods.*

Serum hepcidin was measured by SELDI-TOF-MS (see *Online Supplementary Methods*).

We identified seven patients from four unrelated families homozygous or compound heterozygotes for mutations in the *TMPRSS6* gene. Mutations were either missense or frameshift and two were novel (*Online Supplementary Table S1*). Details about the mutations described are in the *Online Supplementary Methods*. Three families were of Turkish origin, one was Kurdish, consanguinity was reported in three. All patients displayed the characteristic phenotype of IRIDA, with hypochromic microcytic anemia, low transferrin saturation and normal/high serum hepcidin values. Anemia in all probands was first diagnosed in infancy. During follow-up most of them required iron treatment, were unresponsive to oral iron and showed only a partial response to parenteral iron administration (*Online Supplementary Table S1*). Thus, the diagnosis of IRIDA occurred during early childhood, confirming that the condition is not recognized until a routine laboratory screening, because of the normal growth and development of the affected individuals.[9] Moreover we diagnosed two adult IRIDA patients (CII2, CII3 in Figure 1). They had a history of refractory anemia in spite of oral iron therapy. CII2 had a history of oral iron supplementation only during pregnancy, and her molecular diagnosis of IRIDA was only made during the investigation of her son (CIII1).

For four probands (AII3, BII3, CIII1 and DII1) we succeeded in collecting the complete blood count (CBC) performed in the first days of life (Table 1). Furthermore none of the probands had infections during the time in which CBC samples were taken. As reported in Table 1 patients showed normal-borderline hemoglobin (Hb), normal mean corpuscular volume (MCV) and normal mean corpuscular hemoglobin (MCH) indicating that the phenotype of IRIDA was not present at birth. Unfortunately iron parameters (serum iron, transferrin saturation and serum ferritin as well as hepcidin levels) were not performed, since they are not required for healthy neonates. Normal erythrocyte morphology was also documented by the examination of the peripheral blood smear in patient AII3 at 2 days old (Figure 2B). These findings, along with reports of normal birth weights for all these patients, suggest that *in utero* iron transfer was normal, with the depletion of iron stores occurring only after birth. In Figure 2A we show a time

Table 1. Hematological parameters of IRIDA patients in the neonatal period.

					Reference values					
					1-3 days		1 week		2 weeks	
	A II3	B II3	C III1	D II1	mean	±SD	mean	±SD	mean	±SD
Hb, g/dL	13.8	12.5	17.3	13.8	17.3	1.9	16.7	2.2	15.9	1.9
MCV, fL	102	106.8	111.3	101.8	109.1	4.8	107.7	6.2	106.4	3.9
MCH, pg	34.8	33.3	36.1	34.3	34.1	1.3	33.8	1.9	33.7	1.5
MCHC, g/dL	33.8	31.2	32.4	33.7	31.3	0.9	31.6	1.3	31.7	0.9
RDW, %	14.9	15.6	20.4	17.4	16.2	1.1	15.6	1.1	15.5	0.9
RBC, $*10^6/\mu L$	3.9	3.3	4.8	4.02	5.1	0.6	4.9	0.7	4.7	0.6
WBC, $*10^3/\mu L$	15.7	14.5	16	6.7	11.8	3.2	10.8	2.5	11	2.4
PLT, $*10^3/\mu/L$	452	420	236	301	287	88	306	101	420	122

Data were collected from records for A II3 at 2 days, B II3: 2 weeks, C III1: 1 day, D II1: 1 week; reference values of 204 healthy neonates were collected by Dr Yilmaz-Keskin at Samsun Education and Research Hospital in Samsun, Turkey. Details in Online Supplementary Table S3. CBC testing was performed by the Mindray BC-6800 analyzer for CBC testing. Birth weight was within normal range for all probands except for C III1 who was prematurely born.

course of hematological findings (Hb, MCV, MCH and MCHC) of proband AII3 in whom an IRIDA phenotype appeared at the age of 2 months. Also in *Online Supplementary Table S2* we report the time course of iron indices for proband AII3. Suspicion of IRIDA usually occurs during a routine pediatric evaluation. However, in some patients, the condition is recognized only in adulthood, either because their anemia is mild or because it has been misclassified.[9] Remarkably, despite congenital and severe iron deficiency, long-term follow-up of the affected subjects has shown normal growth and intellectual development with no evidence of the cognitive concerns on which iron deficiency screening in infancy have been founded. In healthy fetuses and neonates in mice, because of the rapid growth and expansion of the red cell compartment, hepcidin gene expression is drastically repressed.[10] Very recently, Willemetz *et al*. demonstrated that in *Tmprss6*$^{-/-}$ fetuses, liver *Hamp1* mRNA expression was up to 60 times higher compared with control mice, in which hepcidin expression was only barely detectable.[11] It is noteworthy that *Tmprss6*$^{-/-}$ fetuses and newborns had a lower iron content, mean corpuscular erythrocyte volume (MCV) and hemoglobin (Hb), indicating microcytic anemia secondary to iron deficiency in mutant mice. These observations suggest that, at variance with humans, in mice Mt-2 is required for hepcidin repression during fetal and postnatal development, and its deficiency leads to microcytic anemia *in utero* and at birth, with persistence into adulthood. Moreover, female Tmprss6 homozygote knockout mice are infertile, reflecting yet another difference in the phenotypes of humans *versus* mice with Tmprss6 deficiency.[5] So despite animal models providing a useful genetic model for the analysis of molecular mechanisms that underlie human hematologic disorders, the different IRIDA phenotypes at the neonatal period, as well as the rescue of anemia and alopecia in Tmprss6 mutant mice by iron administration, confirm that the iron regulation and the pathophysiology of the disorder in humans are more complex.

Our findings are further supported by our recent publication on a Turkish female infant who had a molecular diagnosis of IRIDA identified through a family screening at the age of 3 months before she developed an overt IRIDA phenotype.[12] At birth her weight was appropriate for the gestational age. The follow-up data of the same infant were later reported, showing that a typical IRIDA phenotype became evident at 4 months of age.[13]

In this paper we describe for the first time the hematological parameters in the neonatal period of four IRIDA patients (Table 1). Data indicate that anemia is not present *in utero* and at birth and develops during the first months of life. In full-term infants the iron stores, released during the hemolysis of senescent RBCs, support the iron needs of the expanding erythropoiesis and growth until 4-6 months of age[14] when the clinical IRIDA phenotype usually became evident. Follow-up of the patient AII3, from birth until 18 months confirms this observation (Figure 2A). Indeed clinical phenotype in the proband manifests after two months of age, and this probably depends on different genotypes or other environmental factors. Maternal iron status accounts for only 6% of the variation in infant iron stores at birth, and the remaining causes of the highly variable size of the birth endowment are not known, but likely include low birth weight, intrauterine growth retardation, prematurity, the time of cord clamping, maternal smoking, and diabetes in pregnancy.[15] A proof of concept is that maternal-fetal iron delivery in IRIDA probably is not inadequate, as demonstrated by the normal findings in CIII1 who had an affected mother. Moreover this patient (CII2) was the first reported case of a pregnancy of an affected IRIDA woman. This is of note because as iron homeostasis develops during the period of 6-9 months, MT-2 is not essential in hepcidin regulation.[15]

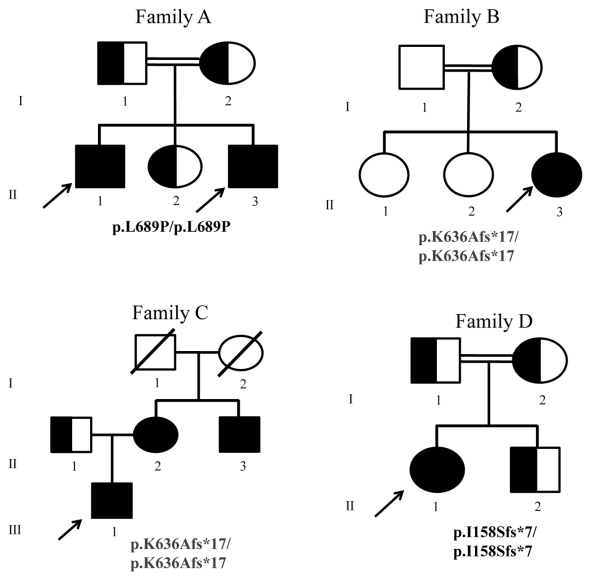

Figure 1. Family pedigree of the affected subjects. *TMPRSS6* mutations identified by automated sequencing are displayed under the pedigree: open symbols, not affected; closed symbols, affected; the half-filled black symbols denote unaffected carriers. Mutations are indicated for each family, mutations in grey have been previously reported. BI1 was not available for genetic studies. The probands are indicated with an arrow.

In conclusion, our study indicates that in humans MT-2 is dispensable during fetal life, which leads to the temptation to speculate that another hepcidin suppressor is produced during fetal development or hepcidin is overexpressed, but because humans are typically born with substantial iron stores, the overexertion of hepcidin is insufficient to actually cause iron deficient erythropoiesis at the time of birth. Due to the nature of the disease the reported numbers are very low, but our results could help produce a better understanding of the role of TMPRSS6, and definitely highlights the differences between men and mice.

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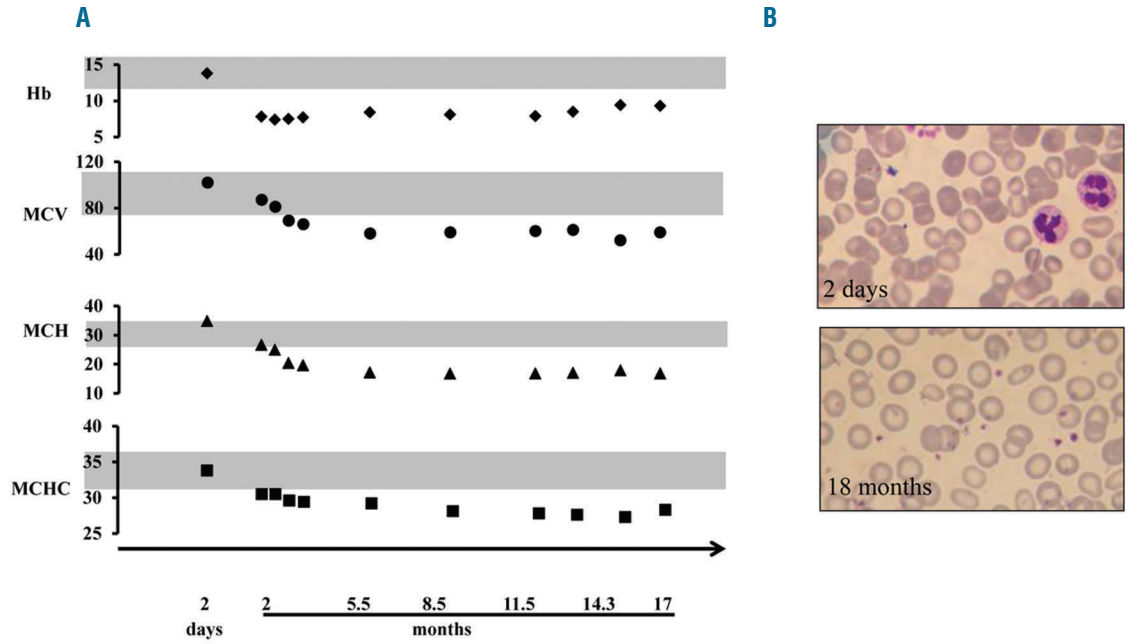

Figure 2. Hematological parameters of patient AII3 during postnatal development. Time course of hematological findings of proband AII3 in the perinatal period (A). Reported on the y-axis are hematological data hallmarked by the IRIDA phenotype: hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), on the x-axis is the age of the patient. Horizontal grey bars indicate normal values. Peripheral blood smears of proband AII3 at 2 days (top panel) and 18 months (bottom panel) of age (B). At 2 days there are no signs of hypochromic microcytic anemia, at 18 months the peripheral smear shows hypochromic cells.

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The online version of this letter has a Supplementary Appendix.

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