Summary

Microcytic hypochromic anaemias are a result of defective iron handling by erythroblasts that decrease the haemoglobin content per red cell. Recent advances in our knowledge of iron metabolism and its homeostasis have led to the discovery of novel inherited anaemias that need to be distinguished from common iron deficiency or other causes of microcytosis. These atypical microcytic anaemias can be classified as: (i) defects of intestinal iron absorption (ii) disorders of the transferrin receptor cycle that impair erythroblast iron uptake (iii) defects of mitochondrial iron utilization for haem or iron sulphur cluster synthesis and (iv) defects of iron recycling. A careful patient history and evaluation of laboratory tests may enable these rare conditions to be distinguished from the more common iron deficiency anaemia. Molecular studies allow distinction of the different types, a prerequisite for differentiated therapy.

Keywords: anaemia, iron deficiency, iron overload, haem, sideroblast, hepcidin.

Common and atypical microcytic anaemias

Microcytosis is a laboratory term that indicates red cells of decreased size and is inevitably accompanied by hypochromia, a sign of low erythrocyte haemoglobin (Hb) content. This definition has a wide clinical use as it denotes distinct types of anaemia, known as microcytic/hypochromic anaemias. Decreased Hb production per cell results from deficiency of either haem or globin chains. In turn reduced Hb content is associated with microcytosis, which is regarded as the consequence of an increased number of cell divisions in an effort to achieve normal Hb content. However, direct proof of this mechanism is lacking.

Iron deficiency anaemia (IDA) is the most frequent acquired microcytic anaemia, common in individuals with high iron requirements as it occurs in infancy, during the growth spurt in adolescents, in menstruating females and in pregnancy. Low dietary iron intake, malabsorption and chronic blood losses are the commonest causes of microcytic anaemia. Furthermore, all of the thalassaemia syndromes (alpha, beta-thalassaemia and the thalassaemic haemoglobinopathies) lead to decreased Hb per cell because of the deficiency of one of the two types of globin chains that assemble to form the Hb tetramer (Hb A) of adult life. Hereditary sideroblastic anaemia, another example of microcytic red cells, is traditionally known to be result of a deficiency of haem synthesis and characterized by the morphological feature of ringed sideroblasts, i.e. erythroblasts with mitochondrial iron deposition visible at Perl’s staining of bone marrow smears. Common causes of microcytic anaemias will not be discussed here.

In recent years, following spectacular advances in our understanding of iron metabolism with the identification of several new proteins involved in iron trafficking and regulation (Hentze et al, 2010), novel forms of microcytic anaemia have been identified. These hereditary anaemias share features with the classic type of IDA. However, personal and family history, complete evaluation of haematological and iron parameters, and careful assessment of the response to treatment may allow their distinction and also provide insights into the defect type.

This article will review rare and recently identified inherited microcytic anaemias, here defined as ‘atypical’, because they differ from the classic IDA or from the classical form of X-linked sideroblastic anaemia caused by mutations of the ALAS2 gene, which encodes the first enzyme of the erythroid haem biosynthetic pathway, delta-aminolevulinic-acid synthase-2 (ALAS2) (Camaschella, 2008; Fleming, 2011). The newly defined forms of microcytic anaemias include defects of iron absorption, transport, utilization and recycling (Table I). For diagnostic purposes, anaemias accompanied by iron overload can be also classified as non-sideroblastic and sideroblastic (Iolascon et al, 2009).

Defects of iron uptake by the transferrin-transferrin receptor 1 (TF-TFR) cycle

Efficient iron uptake by the erythroblasts requires appropriate saturation of transferrin (which approximates 30% in
normal subjects) and a functional transferrin receptor 1 (TFR, also known as TFRC) cycle. The latter is important in all cells, but critical for erythropoiesis. There are no reported defects of the TFRC gene that cause anaemia in humans. Mice in which the Tfrc gene has been constitutively inactivated die of severe anaemia and central nervous system abnormalities during embryonic life (Levy et al, 1999). The relevant role of the TFR cycle in erythropoiesis is also supported by the severe anaemia presented by individuals with hypotransferrinaemia and with mutations of proteins that participate in the TFR cycle, such as solute carrier family 11 member 2 (SLC11A2) also known as proton-coupled divalent metal ion transporter 1 (DMT1) (Iolascon et al, 2008) and 6-transmembrane epithelial antigen of the prostate 3 (STEAP3) (Grandchamp et al, 2011): these forms have been documented both in humans and in animal models (Fleming et al, 1997, 1998; Ohgami et al, 2005).

The common pathophysiology shared by all these conditions is the iron deficiency of the erythron, caused by the low release of transferrin bound iron to maturing erythroblasts. Paradoxically, all these conditions are characterized by iron overload, because iron deficient erythropoiesis provides a false signal of body iron deficiency that suppresses hepcidin through the function of the physiological ‘erythroid regulator’, whose precise nature remains unknown and is the subject of extensive investigation (Andrews, 2008).

**Atransferrinaemia**

Hypotransferrinaemia is not a novel disorder but is discussed here because it is a defect of the TFR ligand transferrin, the serum iron binding protein that releases iron to the tissues. Diferric transferrin binds to TFR at high affinity and releases iron within the acidic environment of the endosome. True atransferrinaemia is probably incompatible with life. Hypotransferrinaemia is an extremely rare autosomal recessive disorder, due to mutations of the TF gene and characterized by very low transferrin levels and severe microcytic hypochromic anaemia from birth. The few patients reported in the literature have high serum iron, undetectable or low levels of fully saturated transferrin, high serum ferritin and signs of iron overload (Hayashi et al, 1993; Aslan et al, 2007; Trombini et al, 2007; Shamsian et al, 2009). Lack of transferrin results in hepcidin suppression and systemic iron overload. Iron is present in the circulation as non-transferin bound iron (NTBI), a highly toxic iron species, easily taken up through still uncharacterized pathways by parenchymal cells including hepatocytes, pancreatic islets and cardiomyocytes.

### Table I. Classification of inherited atypical microcytic anaemias.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>OMIM</th>
<th>Defect</th>
<th>Treatment options</th>
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<tbody>
<tr>
<td>Defective iron absorption</td>
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<tr>
<td>TMPRSS6 defects – IRIDA #206200</td>
<td>Microcytic anaemia Iron deficiency</td>
<td>Hepcidin excess</td>
<td>Parenteral iron</td>
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<tr>
<td>Defective iron transport/uptake</td>
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<tr>
<td>Atransferrinaemia #209300</td>
<td>Microcytic anaemia Iron overload</td>
<td>Defective iron carrier</td>
<td>Plasma infusions</td>
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<tr>
<td>SLC11A2 mutations #206100</td>
<td>Microcytic anaemia Iron overload</td>
<td>Defective endosomal iron release</td>
<td>ESA</td>
</tr>
<tr>
<td>STEAP3 mutations* NA</td>
<td>Microcytic anaemia Iron overload</td>
<td>Defective endosomal iron reduction</td>
<td>Blood transfusions + Iron chelation</td>
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<tr>
<td>Defective mitochondrial iron utilization</td>
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<tr>
<td>X-linked sideroblastic anaemia (XLSA)</td>
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<tr>
<td>XLSA #300751</td>
<td>Microcytic anaemia Iron overload</td>
<td>Defective haem production</td>
<td>Pyridoxine Iron depletion</td>
</tr>
<tr>
<td>XLSA/ataxia 301310</td>
<td>Mild microcytic anaemia Cerebellar ataxia</td>
<td>Defective Fe/S cluster metabolism</td>
<td>None</td>
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<tr>
<td>Autosomal recessive sideroblastic anaemia</td>
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<tr>
<td>SLC25A38 mutations* #610819</td>
<td>Microcytic anaemia Iron overload</td>
<td>Defective mitochondrial glycerophosphate metabolism</td>
<td>Blood transfusions + Iron chelation/ABMT</td>
</tr>
<tr>
<td>GLRX5 mutations* #609588</td>
<td>Microcytic anaemia Iron overload</td>
<td>Defective Fe/S cluster metabolism?</td>
<td>Blood transfusions + Iron chelation</td>
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<tr>
<td>Autosomal dominant</td>
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<tr>
<td>Defective iron recycling</td>
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<tr>
<td>Aceruloplasminaemia #604290</td>
<td>Micro-normocytic anaemia Diabetes, neurodegeneration</td>
<td>Decreased iron release to plasma</td>
<td>None (low dose iron chelation experimental)</td>
</tr>
</tbody>
</table>

OMIM, on line Mendelian Inheritance in Man reference number; IRIDA, iron refractory iron deficiency anaemia; ESA, erythropoietic stimulating agents; Fe/S, iron sulphur cluster; ABMT, allogeneic bone marrow transplantation; NA, not applicable; †, unknown.

*Single family reported.
Studies in the spontaneous hypotransferrinaemic (hpx) mouse, which has strongly reduced transferrin levels because of a splicing defect of the Tf gene (Trenor et al, 2000), show that transferrin is a positive modulator of hepcidin in both an erythropoiesis dependent and independent manner (Bartnikas et al, 2011; Bartnikas & Fleming, 2012).

**Defects of SLC11A2 (DMT1)**

Among the proteins of the TFR cycle, molecular defects of SLC11A2 lead to microcytic hypochromic anaemia. SLC11A2 is a plasma membrane proton-coupled divalent metal transporter, member of the Natural Resistance-Associated-Macrophage Protein (Nramp) family, with an important role in erythroblasts, duodenal cells and macrophages. The SLC11A2 gene, encoding DMT1, was first identified in rodents and cloned in the nk mouse, a model with severe iron-deficient anaemia due to a missense (G185R) homozygous mutation in a transmembrane domain of the gene (Fleming et al, 1997). The same mutation is present in the Belgrade rat, which shows an identical phenotype (Fleming et al, 1998). Iron incorporation into Hb is defective in these animals that are refractory to oral or intravenous iron, the latter finding indicating that the molecular defect is not limited to iron absorption.

Patients with SLC11A2 mutations have microcytic hypochromic anaemia that, in some cases, is present at birth. However, the function of the mutated SLC11A2 may be either reduced or absent thereby influencing the severity of anaemia. In some cases the condition may escape diagnosis until adulthood (De Falco et al, 2012). Patients have increased transferrin saturation and high serum ferritin in the presence of variable degrees of microcytic anaemia. In all studied cases hepcidin is abnormally low relative to iron overload, which develops slowly. Interestingly, liver function tests are normal and fibrosis is not reported in patients who undergo liver biopsy. The development of iron overload strengthens the notion that SLC11A2 is dispensable for intestinal iron absorption in human. One interpretation is that iron in the gut is taken up in the form of haem that can compensate for the inefficiency of the non-haem iron pathway.

Erythropoietin administration ameliorates anaemia in patients by expanding erythropoiesis, without improving the utilization of erythroid iron, as shown by unmodified erythrocyte size and Hb content before and after therapy (Iolascon et al, 2006). A recent study of mice and human erythroid cultures shows that erythropoietin inhibits apoptosis of Slc11a2-mutant erythroid progenitors and differentiating erythroblasts, thereby reducing ineffective erythropoiesis (Horvathova et al, 2012).

**Defects of STEAP 3**

Hypochromic microcytic anaemia due to the deficiency of STEAP 3, a ferrireductase active in the TFR endosomal cycle, was reported in the nm1054 mouse (Ohgami et al, 2006) but not in patients until recently. Three siblings of Pakistani origin with transfusion-dependent hypochromic-microcytic anaemia and iron overload were reported to harbour a nonsense heterozygous (p.Cys100Stop) mutation in the paternal STEAP3 (TSAp6) gene (Grandchamp et al, 2011). In this family no causal mutation was inherited from the normal mother. This puzzling case was interpreted as an example of combination of a mutated allele and a weakly expressed (hypomorphic) allele. To add further complications, the presence of some ringed sideroblasts suggested features common to congenital sideroblastic anaemia, although the mechanism leading to mitochondrial iron deposition remains unexplained.

**Defects of iron absorption**

**Iron refractory iron deficiency anaemia (IRIDA)**

Iron refractory iron deficiency anaemia is a recently recognized recessive disorder that causes microcytic hypochromic anaemia. It is due to mutations of the transmembrane protease serine 6 (TMPRSS6) gene, which encodes matriptase-2, a type II trans-membrane serine protease mainly expressed by hepatocytes (Velasco et al, 2002). The gene was cloned in mask mice, a product of N-ethyl-N-nitrosourea (ENU) mutagenesis, which had microcytic anaemia and a truncated matriptase-2 devoid of the catalytic domain (Du et al, 2008). Tmprss6 knockout mice have a similar phenotype (Folgueras et al, 2008); a few days after birth these mice develop moderate anaemia, lose trunk hairs and show decreased iron absorption because their high hepcidin levels block ferropor-tin-mediated iron release to plasma. In cell models this severe protease cleaves the BMP coreceptor haemojuvelin, attenuating the BMP-mediated hepcidin activation (Silvestri et al, 2008). Matriptase-2 is the first hepcidin inhibitor with a documented in vivo effect in patients.

Although epidemiological data are lacking, IRIDA is considered the most frequent among the rare microcytic anae-mias. Diagnosis of IRIDA usually occurs in childhood. Whereas anaemia is mild-moderate and growth and develop-ment are normal, microcytosis and hypochromia are remark-able and disproportionate to the degree of anaemia (Finberg et al, 2008). Transferrin saturation is extremely low, while ferritin may be normal or at least not as low as one would expect from transferrin saturation. From the limited follow-up data, anaemia seems to be more severe in children than in adults. In the affected individuals, who belong to a large kindred originating from and living in a small Sardinian vil-lage (Melis et al, 2008), adult patients show mild anaemia, but persistently low transferrin saturation and remarkable microcytosis. Hepcidin levels are high/normal when mea-sured in patient serum or urine, in any case inappropriate to iron deficiency, given that in the latter condition hepcidin expression is strongly suppressed.
Patients from about 50 unrelated families of different ethnic origin have been described as being either homozygous or compound heterozygous for TMPRSS6 mutations (Camashella & Poggiali, 2011), predicted to result in loss of function of the encoded protein. Missense mutations are spread along the entire gene sequence, affecting not only the protease catalytic domain, but also other domains that could affect protein–protein interaction (Finberg et al, 2008; Guillem et al, 2008; Melis et al, 2008; Silvestri et al, 2009; Ramsay et al, 2009; De Falco et al, 2010; Altabara et al, 2010; Guillem et al, 2012). Most mutations are private. In vitro studies have shown that causal mutations have decreased activity and are unable to inhibit hepcidin promoter at the same rate of the wild type protein in a luciferase-based assay in cells transfected with haemojuvelin (Silvestri et al, 2009; Ramsay et al, 2009; De Falco et al, 2010). To date, there is no evidence of genetic heterogeneity of IRIDA. Only homozygous TMPRSS6 mutations have been found in a few patients, although regulatory regions are not usually explored by sequencing (Finberg et al, 2008). It is possible that single nucleotide polymorphisms (SNPs) or specific haplotypes play some role in the disease (Kloss-Brandstatter et al, 2012; Delbini et al, 2010), and that cases showing microcytosis without anaemia are due to mild TMPRSS6 mutations. 

Animal models of microcytic anaemia not reported in humans

Based on animal models of decreased iron absorption and defective iron uptake other disorders might be hypothesized, though not yet reported, to cause microcytic anaemia in patients. 

Mutations of hephaestin, an oxidase involved with ferroportin in iron export from the basolateral membrane of the absorptive enterocytes, cause severe IDA in Sla mice. In this model iron, retained in the enterocytes, is lost from the body with duodenal mucosa desquamation (Vulpe et al, 1999).

Gastrin $\sim/-$ mice have normal haematological parameters in basal conditions, but are unable to recover from established iron deficiency (Kovac et al, 2011). Having reduced basal gastric acid secretion they develop severe anaemia, splenomegaly and thrombocytosis and do not recover after oral iron treatment. 

Iron absorption requires HCl and low pH. The sublytic mouse is an ENU-induced mutant animal with achlorhydria that results from a homozygous mutation in the ATPase $\gamma$ (Atp6v1a) gene, encoding a subunit of the gastric hydrogen-potassium pump (Krieg et al, 2011). Anaemia in this model is corrected by parenteral iron administration or by acidification of drinking water. This model indicates that in rodents the gastric proton pump is essential for iron absorption and effective erythropoiesis. However, as humans differ from mice regarding haem absorption (Iolascon et al, 2006) it is unlikely that the latter mice are models of human macrocytic anaemia.

Haemoglobin deficient (Hbd) mice carry a spontaneous in-frame deletion of an entire exon of the Exoc6 (Sec15I) gene, a defect that impairs erythroid iron utilization (Lim et al, 2005). EXOC6 interacts with Rab11, a GTPase involved in vesicular trafficking. In yeast, the Exoc6 homologue protein is a member of the exocyst family (White et al, 2005) that is probably involved in sorting the TF-TFR complex to the recycling endosomes (Garrick & Garrick, 2007). No human counterpart of this defect has been described.

Defects of mitochondrial iron utilization

Iron is mainly utilized in the mitochondria to be incorporated into protoporphyrin IX for haem formation by ferrochelatase and for iron sulphur cluster biogenesis [for review see (Hentze et al, 2010; Rouault, 2012)]. Sideroblastic anaemia includes a heterogeneous group of disorders characterized by iron accumulation in mitochondria of erythroblasts. Because of the perinuclear disposition of the organelles this accumulation gives rise to the morphological feature of ‘ringed sideroblasts’. The disorders may be genetic or acquired. Hereditary sideroblastic anemias are considered as disorders of haem synthesis but in broad terms they may be included in iron-related anemias, because some recently identified forms have defects related to iron-sulphur cluster biogenesis. Discussing syndromic forms of sideroblastic anaemia, such as Pearson syndrome, Kearns-Sayre syndrome or thiamine-responsive megaloblastic anaemia, is out the scope of this review. Similarly, acquired forms secondary to drugs, such as pyridoxine antagonists (isoniazid, cycloserine) and toxins (lead, ethanol) and the Refractory Anaemias with Ringed Sideroblasts (RARS) that are considered clonal myelodysplastic disorders, are not discussed here, as they are usually normo or macrocytic (May, 2011).

The type of inheritance of sideroblastic anaemia is variable, including X-linked, recessive and dominant inheritance (Table I). The X-linked form is well established; recently the
genetic cause of recessive forms has been clarified, whereas that of the dominant forms remains unknown.

**X-linked sideroblastic anaemia**

The X-linked form is the most common type and affects primarily males. It is due to mutations of ALAS2, the first enzyme of the haem synthetic pathway. Anaemia is present in the first months of life, but diagnosis may occur in midlife or in the elderly in benign cases. This form has been extensively discussed in several reviews (Bottomley, 2006; Camaschella, 2008; May, 2011; Fleming, 2011). Because of the X-linked inheritance, males are usually affected but anaemia can be present also in females due to skewing of X chromosome inactivation. The age of onset is variable. The disease is less severe than the recessive form and transfusion-dependency is unusual. The ALAS2 mutations are prevalently missense and affect mainly the catalytic domain (Bottomley, 2006). Mutations close to the 5′pyridoxal phosphate binding site confer responsiveness to pyridoxine, whereas carriers of other mutations are usually pyridoxine-refractory (Bottomley, 2006). The severity of anaemia may be modified by other genetic or acquired factors, such as HFE C282Y mutations or iron overload (Camaschella, 2008).

Another form that affects males is X-linked sideroblastic anaemia/ataxia, a rare anaemia due to defects (all missense mutations) of the ATP-binding cassette B7 gene (ABCB7), whose product is thought to be responsible for the iron sulphur cluster export from mitochondria to the cytosol. Few patients have been reported (Bekri et al, 2000) with early onset of cerebellar ataxia. The anaemia is mild, red cells are hypochromic and microcytic and variable percentages of ringed sideroblasts are found in the bone marrow. However, iron overload has not been reported. The mechanism of ring sideroblast formation is unclear. It is possible that ABCB7 deficiency impairs the activity of ferrochelatase, the last enzyme of haem synthesis, which is iron/sulphur-dependent (Cavadini et al, 2007). This would establish a link between the two pathways of mitochondrial iron utilization.

**Autosomal recessive and other forms of sideroblastic anaemia**

An autosomal recessive form affects SLC25A38, a putative amino acid transporter of the inner mitochondrial membrane, potentially involved in mitochondrial import of glycine, a substrate essential for delta-aminolevulinic-acid (ALA) synthesis. SLC25A38 mutations cause severe microcytic/hypochromic sideroblastic anaemia in different populations. Males and females are equally affected. The degree of anaemia is more severe than that caused by ALAS2 mutations and usually results in transfusion dependency (Guernsey et al, 2009). Anaemia is of early onset being present at birth or during the first months of life (Guernsey et al, 2009; Bergmann et al, 2010). Homozygous or compound heterozygous subjects have been identified with different (premature termination, splicing alteration or missense) substitutions in conserved amino acids (Guernsey et al, 2009; Kannengiesser et al, 2011; Fleming, 2011).

A single patient from a consanguineous family was reported with recessive sideroblastic anaemia resulting from a glutaredoxin 5 (GLRX5) splice homozygous mutation. The patient was diagnosed in midlife with severe anaemia, heart failure, cirrhosis and diabetes secondary to iron overload. The substitution strongly decreases the corresponding GLRX5 RNA and protein (Camaschella et al, 2007). The increased IRP1 activity, in the absence of the iron/sulphur clusters-dependent conversion of IRP1 to aconitase, represses ALAS2 translation resulting in haem deficiency and anaemia. This interpretation is in keeping with the data obtained in the zebrafish mutant shiraz, a spontaneous model of Glrx5 deficiency (Wingert et al, 2005) and was confirmed in patient cells (Ye et al, 2010). Recently a second case, compound heterozygous for two missense mutations, was identified in a Chinese patient with a similar phenotype (Guangjung Nie, CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, National Centre for Nanoscience and Technology, Beijing, China, personal communication) The anaemia improved following long-term iron chelation and both patients became transfusion-independent.

Defects of ABCB7 and GLRX5 are examples of congenital sideroblastic anaemia that result from defects in proteins involved in iron/sulphur cluster transport and biogenesis respectively.

Other forms of sideroblastic anaemia are autosomal dominant (Bottomley, 2006). Proposed candidate genes for these forms and for families in which mutations have not been found are enzymes of the iron sulphur cluster assembly (May, 2011).

Erythropoietic protoporphyria is an autosomal dominant disorder due to mutations of the gene (FECH), which encodes ferrochelatase, the last enzyme of haem biosynthesis. The penetrance is incomplete because a hypomorphic allele that is common in the general population may be in trans to a rare loss-of-function mutation of FECH. The disease is characterized by photosensitivity due to accumulation of protoporphyrin IX and is classified under the porphyrias. However, mild microcytic anaemia occurs in 20–60% of patients with some evidence of iron deficiency (Holme et al, 2007). Studies in a mouse model of this complex disorders indicate that the iron deficiency is spurious as a result of excessive transferrin synthesis (Lyoumi et al, 2007).

Interestingly, some mutations in the C-terminal region of ALAS2 generate dominant erythropoietic protoporphyria instead of sideroblastic anaemia (To-Figueras et al, 2011).

**Defects of iron recycling**

**Aceruloplasminaemia**

Approximately 20–25 mg of iron are recycled daily by macrophages, which destroy senescent erythrocytes. Iron is
rapidly released by haem oxygenase and recycled through ferroportin to plasma. This transfer in macrophages requires the oxidative function of ceruloplasmin, a plasma protein secreted by the liver. For this reason anaemia occurs in the rare recessive aceruloplasminemia, which results from ceruloplasmin (CP) gene mutations (Yoshida et al, 1995). CP ferroxidase activity is essential to export iron from macrophages, hepatocytes and neuronal cells and to release it to transferrin in the form of ferric iron. Studies in patients and in the Cp knockout mouse revealed increased lipid peroxidation due to iron-mediated reactive oxygen species injury. Aceruloplasminemia is a syndrome, classified under a disorder called ‘neurodegeneration with brain iron accumulation’ (NBIA) (Kono, 2012). Affected subjects may present the clinical triad of retinal degeneration, diabetes mellitus and neurological disease (ataxia, movement disorder and cognitive dysfunction). However, this occurs only in midlife and diagnosis may be difficult in the early stages. Recognition of anaemia may help in the diagnosis because normochromic normocytic or slightly microcytic anaemia of mild-moderate degree (and diabetes) (Ogimoto et al, 2011) appear earlier than neurological symptoms. Anaemia, low transferrin saturation and low serum copper co-exist with high serum ferritin. Serum CP is undetectable. Serum hepcidin was measured in a few cases and was found to be low (Kaneko et al, 2010). Studies in vitro and in knock-out mice established that iron export is decreased in hepatocytes, macrophages and glial cells, that use CP as the oxidase essential for iron export through ferroportin (Harris et al, 1999).

Pathophysiology of microcytosis

Is there a common mechanism that in all the above described anemias leads to small red cell size and decreased Hb content? A common feature is decreased haem formation. It is well known that haem is a powerful controller of different cell functions. Among others, haem regulates a kinase (Haem-regulated eIF2alpha kinase or HRI), which is highly expressed in erythroid cells, that phosphorylates the alpha-subunit of the eukaryotic translational initiation factor 2 (eIF2alpha). When haem concentration declines, HRI blocks the kinase, thereby preventing further protein synthesis. Globin is the main protein synthesized by erythroblasts: that is why translational control by haem ensures the needed balance between globin and haem synthesis. HRI has an essential protective role in iron deficiency, avoiding excessive protein synthesis and is responsible for hypochromia as an adaptive mechanism for erythrocyte survival (Chen, 2007).

It has also been proposed that the erythrocyte size depends on the total iron present in the erythroid precursors and especially on the ability of red cell precursors to release iron through ferroportin B, a ferroportin isoform lacking the 5′ UTR IRE element at the RNA level, and thus unresponsive to iron (Zhang et al, 2009, 2011) but still responsive to hepcidin. According to some authors, low hepcidin in IDA would allow ferroportin to export iron from red cell precursors leading to microcytosis, whereas high hepcidin in anaemia of chronic disease (ACD), which is characterized by iron-restricted erythropoiesis, would cause iron retention in red cell precursors and lack of microcytosis (Keel & Abkowitz, 2009).

Pathophysiology of iron overload

A common mechanism leads to increased iron absorption in all cases that develop iron overload because of the activation of the ‘erythroid regulator’ that drives extra iron supply to erythropoiesis. In defects of iron transport and of TFR cycle erythropoiesis is iron-deficient, while in sideroblastic anaemia reduced iron utilization and ineffective erythropoiesis trigger increased absorption. Although the nature of the ‘erythroid regulator’ remains to be defined, its activity converges on the same hepcidin inhibitory pathway and is common to these and other iron loading anemias (Papanikolaou et al, 2005; Tamary et al, 2008; Origa et al, 2007; Casanovas et al, 2011). Among signals released from erythroid cells, the transforming growth factor-beta family member GDF15 has been claimed to mediate hepcidin inhibition (Tanno et al, 2007). Although increased in conditions of ineffective erythropoiesis and often found inversely related to hepcidin levels, GDF15 seems at most a marker of ineffective erythropoiesis but certainly not the sole ‘erythroid regulator’ (Tanno & Miller, 2010). In murine models TWSG1 has been proposed as a candidate for this function (Tanno et al, 2009). Another strong candidate remains the soluble isoform of TFR (sTFR), which is increased both in iron deficiency and in erythropoiesis expansion. Its role has not been evaluated in depth and was probably too rapidly dismissed after studies in transgenic animals (Flanagan et al, 2006). Erythropoiesis is iron-restricted in aceruloplasminemia, but total body iron is not reduced, because iron accumulates in specific districts, such as macrophages, hepatocytes, retinal cells and astrocytes.

Making the diagnosis

Patient history

Personal history of the patient and full family history may be informative. As in all other genetic disorders, extended pedigrees should be examined to identify transmission modalities, whenever possible.

To differentiate between genetic and acquired microcytosis all previous complete blood counts (CBC) should be carefully assessed, with special attention to Hb and erythrocyte indices. Anaemia and microcytosis are present from birth in most cases, with the exception of IRIDA, where birth weight is normal and anaemia develops during the first months of life. On the contrary, SLC11A2 mutations and recessive sideroblastic anaemia should be considered in the differential diagnosis of microcytic anaemia present at birth and in the neonatal period.
History of photosensitivity may address the diagnosis in cases of erythropoietic protoporphyria.

The effect of any previous treatment should be carefully evaluated. To establish refractoriness to oral iron, both the type of prescribed iron and whether the dosage was appropriate and the patient compliant to therapy should be assessed. Positive History of transfusions (even occasional transfusions) should be recorded, as the number of transfused units enables estimation of the amount of extra-iron introduced. In IRIDA patients followed for many years the severity of anaemia seems to decrease with time, in parallel with the reduced iron requirements of adults compared to young individuals (Melis et al, 2008): thus often iron treatment is limited to the childhood period.

**Physical examination**

Apart from pallor, physical examination is usually unremarkable in children. In cases of severe anaemia, growth and development may be delayed. Liver and spleen are usually not enlarged. In adults, according to the type of anaemia, signs of iron overload, such as skin pigmentation and signs of cirrhosis and portal hypertension may be apparent.

**Laboratory tests**

Indispensable tests for diagnosis include CBC, erythrocyte indices and peripheral blood smear. Microcytosis is defined by mean cell volume (MCV) and mean cell Hb (MCH) values that are lower than the age reference values (MCV < 80 fl and MCH < 27 pg in adults). However, these values are definitely quite low, especially in IRIDA where they are around 50–60 fl, both in children and adults (Cammaschella & Poggiali, 2011). Nevertheless, even low-normal or borderline values of erythrocyte indices may be present with abnormal iron parameters (e.g. in aceruloplasminaemia). Usually, the described disorders do not show all the features of IDA. Atypical laboratory parameters can orient the diagnosis. The main differences between iron parameters in these forms in comparison with iron deficiency and ACD are reported in Table II.

Figure 1 illustrates a useful algorithm for diagnosing the atypical microcytic anaemias. After exclusion of common causes of microcytosis, especially the thalassaemia syndromes, it is important to consider the differences in iron parameters.

High serum ferritin and high serum iron in the presence of normal transferrin levels, suggest either transport/uptake (non-sideroblastic) defects or sideroblastic anaemia.

Morphological studies of the peripheral blood smear are useful in sideroblastic anaemia as they may reveal the presence of siderocytes, erythrocytes with coarse granular inclusions (Pappenheimer bodies) positive for iron. A dimorphism of red blood cells may be present that results in increased red cell distribution width (RDW) in sideroblastic anaemia but is absent in the non-sideroblastic form. Bone marrow aspirate or biopsy is usually performed only in cases of severe anaemia or in moderate longstanding anaemia in the absence of a clear diagnosis. However, Perl’s staining of the bone marrow smear is essential to reveal ‘ringed’ sideroblasts and allow definite distinction of sideroblastic and non-sideroblastic anaemia. According to the classical definition, sideroblasts should be at least 15% of total erythroblasts and the perinuclear ring should encompass at least 1/3 of the nucleus rim. The number of sideroblasts is usually higher than the established cut-off, but exceptions exist. Other tests have been proposed, including the assessment of mitochondrial ferritin (Della Porta et al, 2006) by cytofluorographic analysis; however Perl’s staining remains the diagnostic gold standard for this type of anaemia.

| Table II. Variation of iron parameters in the atypical microcytic anaemias compared with ACD and IDA. |
|---------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Family history | Atransferrinaemia | SLC11A2 defects | STEAP3 defects | Sideroblastic Anaemia | IRIDA | ACD | IDA |
| Anaemia at birth | Siblings | Siblings | Siblings | According to Inheritance | Siblings | No | Sometimes |
| Degree of anaemia | Severe | Variable | Severe | Variable | Moderate | Moderate | Variable |
| MCV | Low | Low | Low | Low | Very low | Normal/Low | Variable |
| MCH | Low | Low | Low | Low | Very low | Normal/Low | Variable |
| Serum Iron | High | High | High | High | Very low | Normal/Low | Low |
| Transferrin | Low/Undetectable | Low | Low | Low | Very low | Normal/Low | High |
| Transferrin saturation | High | High | High | High | Normal/Low | Low |
| Serum ferritin | High | High | High | High | Normal/Low | Normal/High | Low |
| sTFR | Low | High | ? | High | High | Low | High |
| LIC | High | High | High | High | Low | Normal/High | Low |
| Serum hepcidin | Low | Low | ? | Low | Normal/High | High |

IDA, iron deficiency anaemia; ACD, anaemia of chronic diseases; IRIDA, iron refractory iron deficiency anaemia; MCV, mean cell volume; MCH, mean cell haemoglobin; sTFR, soluble transferrin receptor; LIC liver iron concentration; ?, unknown.
In the presence of low iron and normal/high serum ferritin, aceruloplasminemia is easily excluded by serum ceruloplasmin level. Diagnosis of IRIDA requires extensive clinical and laboratory investigation to exclude common acquired IDA/microcytosis. Transferrin saturation in untreated cases is extremely low (around 2–5%) and disproportionate to the level of serum ferritin that can be normal or even increased (especially after iron treatment). Diagnosing IRIDA also requires that iron refractoriness is established by means of a trial of oral and i.v. iron (see Treatment).

Serum or urinary hepcidin levels have been measured by different techniques in most of the published cases of IRIDA and in some other rare anaemias. There is increasing interest in tests that measure hepcidin levels, but currently available methods, based on immunochemical or mass spectrometric assays, are designed for research purposes only (Ganz et al., 2008). Recent advances to improve the assays and international standardization efforts (Kroot et al., 2009) look promising for the introduction of hepcidin level measurement in clinics. This possibility would facilitate IRIDA diagnosis in the future because IRIDA patients have normal/high serum hepcidin levels while iron-deficient subjects have consistently very low/undetectable levels. However, inflammatory conditions that may increase the hepcidin production should be excluded by normal C reactive protein levels.

Second level tests include measurement of serum soluble transferrin receptor (sTFR) levels: high levels are found in sideroblastic anaemia, in patients with SLC11A2 mutations and in IRIDA, but not in hypotransferrinaemia (Table II), unless the patient has been treated with plasma or transferrin infusions (Trombini et al., 2007). Liver Iron Concentration (LIC), currently performed by magnetic resonance imaging (MRI), should be used to monitor all forms prone to develop iron overload (St Pierre et al., 2005). In severe iron overload, cardiac iron assessment by T2star is indicated to plan adequate treatment. Liver biopsy or MRI may demonstrate iron overload and brain MRI reveals iron accumulation in basal ganglia in aceruloplasminemia (Kono, 2012).

Molecular tests based on DNA sequencing are used to define these rare atypical forms of anaemia. They are required in all cases for a precise diagnosis, except for atransferrinaemia and aceruloplasminemia that are diagnosed dosing the serum protein levels. The need of gene sequencing can be discussed in sideroblastic anaemia considering that a morphological diagnosis is available. In addition, molecular diagnosis is considered expensive and time consuming and, in some cases, scarcely available. However, molecular diagnosis in these rare conditions may provide useful information in order to better understand the disease phenotype and also to plan the patient treatment. In addition, it allows genetic counselling and family screening and may identify other affected family members, who may be potentially treatable before the development of complications. Still, it should be kept in mind that, because of genetic heterogeneity in most disorders, in several cases genetic tests are diagnostic only when positive. Conceivably, the development of new diagnostic tools in the future will change our diagnostic approach. As an example, the clinical availability of laboratory determination of the hepcidin levels would probably make genetic testing for TMPRSS6 mutations redundant, which is currently required for a precise diagnosis.

**Differential diagnosis**

Differential diagnosis of microcytic anaemia with thalassaemia syndromes is based on clinical observations and specific laboratory tests. The ethnic origin of the family and the Hb analysis can assist in the diagnosis, as beta-thalassaemia carriers are easily recognizable by their increased HBA2 levels. Iron parameters are usually normal/elevated in these individuals but iron deficiency may coexist for other causes. The
degree of anaemia is extremely severe in homozygous beta-thalassaemia and moderate in IRIDA. Patients with transfusion-independent thalassaemia intermedia show jaundice and signs of haemolytic anaemia with enlarged liver and spleen. Hb tests may easily orient the diagnosis. Carriers of alpha thalassaemia may have remarkable microcytosis but usually are not or only mildly anaemic. Differential diagnosis of HbH diseases (usually with enlarged spleen) may require molecular studies.

As in IDA of a comparable degree, erythrocyte zinc protoporphyrin as well as sTFR and platelet count may be increased in IRIDA. However, transferrin saturation is disproportionately low as compared to serum ferritin, and MCV and MCH are remarkably lower than in IDA. The iron absorption test (Finberg, 2009) is rarely performed in clinical practice. It requires the exclusion of acquired intestinal disorders (coeliac disease, inflammatory bowel diseases, Helicobacter pylori infection) for the interpretation of the results (Oustamanolakis et al., 2011; Hershko & Skikne, 2009). However, the iron deficiency resulting from malabsorption for acquired diseases does not show the delayed response to parenteral iron, another distinctive feature of IRIDA (see below). ACD may share features with IRIDA, because of high hepcidin production secondary to cytokine stimulation. In this condition, iron is increased in macrophages and stores but decreased at the erythropoietic level (Weiss & Goodnough, 2005; Goodnough et al., 2010). Microcytosis and hypochromia are usually absent/borderline and transferrin saturation is less decreased unless ACD is longstanding, especially severe or is accompanied by true iron deficiency. Table II shows the main variations of iron parameters among the described anaemias.

**Treatment**

The experience of treatment of these rare disorders is still extremely limited. Thus the following recommendations should be considered the expression of the Author’s view. Atypical microcytic anaemias require precise diagnosis because treatment differs considerably, with some cases requiring iron supplementation or blood transfusions, and others requiring iron chelation or further drugs/approaches (Table I).

Survival in atransferrinaemia requires regular fresh-frozen plasma or purified transferrin infusions. The treatment corrects anaemia and prevents iron overload, as the excess iron is consumed in erythropoiesis and restored hepcidin production blocks the excessive iron absorption (Trombini et al., 2007). Iron chelation is mandatory for established iron overload.

Patients with SLC11A2 defects do not respond to oral or intravenous iron, which can even worsen iron overload. According to the severity of anaemia, blood transfusions are required in the neonatal period and first infancy to allow normal development (Iolascon et al., 2008). In patients requiring transfusions, anaemia may be partially improved by erythropoietic stimulating agents (ESA) that augment total erythropoiesis. Short courses of erythropoietin improve anaemia due to infections and may be utilized in special circumstances, such as in preparation for surgery.

Iron refractory iron deficiency anaemia patients do not respond to oral iron and respond partially and slowly to parenteral iron, in spite of increased ferritin that indicates adequate iron stores. Hb response may occur after a prolonged parenteral treatment that leads to very high ferritin levels, because pharmacological iron accumulates in macrophages as an effect of high hepcidin and is slowly released. At least two iron cycles, one oral and one parenteral, should be completed before concluding that anaemia is iron-refractory. Ascorbic acid (30 mg/d) supplementation at the time of ferrous sulfate administration has been reported to improve Hb and iron status in an infant patient (Cau et al., 2012). Another two patients were treated with ESA plus iron to counteract excessive liver iron deposition caused by parenteral iron treatment and to control anaemia due to hypermenorrhrea (Ramsay et al., 2009).

As for sideroblastic anaemia, patients with ALAS2 mutations occurring close to the piridoxal-5′-phosphate binding site may respond to oral vitamin B6 (pyridoxine). Pharmacological doses (100–200 mg) of vitamin B6 are worth a trial in X-linked sideroblastic anaemia due to ALAS2 deficiency, but are useless in the other forms. Responsive patients must remain on pyridoxine (10–100 mg/d) maintenance life-long: Hb levels do not normalize, but transfusions may be avoided.

Anaemia is severe in SLC25A38 deficiency and requires blood transfusions from infancy. A few cases have successfully received allogeneic bone marrow transplantation (Guernsey et al., 2009). Anaemia is mild and does not require treatment in sideroblastic anaemia with ataxia. In all patients treated with chronic transfusion protocols, but also in untransfused patients with X-linked sideroblastic anaemia (and with GLRX5 deficiency), the iron status should be monitored and appropriate treatment should be started as soon as signs of iron overload become manifest (usually for ferritin levels >1000 μg/l), to avoid iron toxicity-related complications (Angelucci et al., 2008). Oral iron chelators, such as deferasirox, may be used in transfused patients. The use of deferasirox in untransfused patients is off label (or compassionate): the dose should be adjusted to the level of iron overload and the development of complications monitored (Porter et al., 2011). Given that iron depletion by phlebotomy or chelators has partially corrected anaemia in a few patients, this approach is worth trying in iron-loaded patients.

Oral iron is ineffective in aceruloplasminaemia because iron is badly distributed and its release from macrophages is compromised. Both iron chelation and phlebotomy were unsuccessful in anecdotal cases (Kono, 2012; Finkenstedt et al., 2010). Iron chelators can reduce total body iron, but also transferrin saturation and should be used with caution.
because they may worsen the anaemia. Oral iron chelators that cross the blood brain barrier, used at low doses in other neurodegenerative disorders, are novel promising approaches (Boddaert et al, 2007; Abbruzzese et al, 2011).

Research advances continue to improve our knowledge of iron metabolism and its disorders. Therapeutic tools are still limited, but translational effort in combination with improved technology is expected to further clarify the pathophysiology of these disorders, and to provide novel molecular targets in order to improve both their diagnosis and treatment.

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References


Review

Papanikolaou, G., Tzilianos, M., Christakis, J.I., European Journal of Gastroenterology & Hepatology, 37, 1164–1171.


Blackmore, K. (2005) Iron metabolism mutant bbd mice have a deletion in Sec15l1, which has homology to a yeast gene for vesicle docking. *Genomics, 86*, 668–673.


