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ABSTRACT BOOK

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Non-Transfusion Dependent Thalassemia (NTDT)

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Introduction

Non-transfusion-dependent thalassemia (NTDT) describes patients with a genetic defect or combination of defects that affect hemoglobin (Hb) chain synthesis, and consequently, the oxygen-carrying capacity of red blood cells (RBCs). Of the numerous NTDT genotypes, the three most studied are: β -thalassemia intermedia (β -TI), α -thalassemia intermedia (HbH disease) and hemoglobin E/ β -thalassemia, with the latter being by far the most common, accounting for about 50% of moderate to severe cases.

Pathophysiology

Three main factors govern the pathophysiology of β -TI: ineffective erythropoiesis, chronic anemia, and iron overload. The degree of ineffective erythropoiesis is the primary determinant of the development of anemia, whereas peripheral hemolysis of RBCs remains secondary. Furthermore, ineffective erythropoiesis and chronic anemia lead to hepcidin suppression by erythroid factors, increased iron absorption from the gut and increased release of recycled iron from the reticuloendothelial system (RES); all culminating in non-transfusional iron overload, mainly in the liver and less so in the heart. Further iron accumulation may occur with occasional transfusions required in acute stress settings or more frequent transfusions required in growing children or adults with vascular complications.

Clinical Complications

The clinical picture seen in NTDT is due to an interaction of the three aforementioned hallmark pathophysiologic factors; and each is associated with certain morbidities. Ineffective erythropoiesis causes compensatory extramedullary hematopoiesis (EMH) and as such is associated mainly with skeletal complications, while peripheral hemolysis has been more implicated in hypercoagulable complications such as pulmonary hypertension (PHT), with secondary heart failure (HF), and thromboembolic phenomena. Iron overload per se accounts for a significant proportion of morbidity in NTDT. In a recent study of 168 non-chelated patients with NTDT, higher Liver Iron Concentration (LIC) values on magnetic resonance imaging were associated with a significantly increased risk of developing thrombosis, pulmonary hypertension, hypothyroidism, hypogonadism, and osteoporosis. Specifically, LIC levels ≥ 5 mg Fe/g dry weight were associated with a considerable morbidity risk increase. Although LIC assessment remains the gold standard for quantification of total body iron, in resource-poor countries serum ferritin (SF) measurement may be the only method available for the assessment of iron overload. Observational studies confirm a positive correlation between SF level and LIC in NTDT patients. A recent longitudinal follow-up over a 10-year period (ORIENT study) confirmed these findings, demonstrating that the cumulative incidence of multiple morbidities was highest in patients with SF ≥ 800 ng/ml and lowest patients had SF ≤ 300 ng/ml. However, it should be noted, that close to 50 % of patients with a SF level between 300-800 ng/ml may still have an LIC ≥ 5 mg Fe/g dw, highlighting the need for LIC assessment in this subgroup of patients. Without appropriate treatment, the incidence of morbidities increases with advancing age.

Management

Management of NTDT involves four main principles: transfusions, splenectomy, HbF inducers and iron chelation.

Traditionally, **transfusions** were reserved for palliation of late and irreversible anemia-related complications; however, more recent evidence supports an earlier role in NTDT management.

The OPTIMAL CARE study, demonstrated that NTDT patients placed on (intermittent or regular) transfusion regimens suffered fewer complications; mainly EMH, PHT and thrombosis. Thus, there is still merit in considering blood transfusions, with indications including acute stress (Hb level < 5 g/dL, surgery, infection or pregnancy), progressive changes from childhood (declining Hb level in parallel with profound splenomegaly, growth failure, failure of secondary sexual development in parallel with bone age, severe bony changes) and other complications (thrombotic or cerebrovascular disease, PHT, extramedullary hematopoietic pseudotumors, leg ulcers). However, in the absence of randomized trials, there are no specific guidelines on the amount and duration of transfusions needed and thus, therapy should be tailored to individual patient needs.

Splenectomy is common practice in NTDT patients, and serves to increase total Hb level by 1-2 g/dl; however, cumulative evidence confirms an association with a variety of adverse outcomes. The spleen functions to scavenge procoagulant platelets and RBCs, which together are the key factors underlying the hypercoagulable state observed in NTDT. Splenectomized NTDT patients are at increased risk for venous thromboembolism, PHT, leg ulcers and silent cerebral infarction than their non-splenectomized counterparts. Nonetheless, splenectomy may be indicated in certain clinical settings such as: hypersplenism (resulting in worsening anemia, leucopenia or thrombocytopenia or their clinical manifestations), worsening anemia leading to poor growth (when transfusion therapy is not possible) or splenomegaly (accompanied by left upper quadrant pain, early satiety or concern of splenic rupture).

HbF inducers work by increasing γ -globin production, a β -like globin molecule which can bind excess α -chains, thus decreasing the α/β -chain imbalance inherent to thalassemia, and improving effective erythropoiesis. Hydroxyurea (or hydroxycarbamide) has been the most studied HbF inducer in NTDT. Early case reports documented hematological improvements in β -thalassemia patients treated with hydroxyurea, and since then several studies have evaluated this drug in NTDT. However, the data is conflicting and mostly comes from single-arm trials or retrospective cohort studies.

Although HbF inducers are a potentially promising aspect in NTDT treatment, large, randomized, controlled trials are needed before these agents or their derivatives are widely used in management.

Finally, **iron chelation therapy** is currently the cornerstone of managing NTDT patients and minimizing disease related complications.

Three chelators are available to treat iron overload: Deferoxamine, Deferiprone, and Deferasirox. Subcutaneous deferoxamine was the first iron chelator studied in NTDT. The current challenges posed by this drug are two-fold: there is a lack of solid evidence from large studies on the benefit of this drug in NTDT patients, and more importantly, its cumbersome subcutaneous administration may cause compliance challenges. Deferiprone, although orally administered, has only been evaluated in few trials with relatively small sample sizes. Deferasirox on the other hand, has been extensively studied in NTDT and is currently the only iron chelator specifically approved for NTDT patients. This once-daily administered oral iron chelator was the agent used in the largest and first randomized clinical trial of chelation therapy in NTDT (THALASSA). Patients ≥ 10 years of age with LIC ≥ 5 mg Fe/g dw received 12 months of therapy with Deferasirox and a significant reduction of LIC was observed, as compared to placebo. LIC decreased by a mean of 2.33 ± 0.70 and 4.18 ± 0.69 mg Fe/g dw in patients receiving starting doses of 5 mg/kg/day and 10 mg/kg/day, respectively. The efficacy of deferasirox was observed across all evaluated patient subgroups. The study also demonstrated Deferasirox's safety profile was comparable to placebo. An extension of the core study showed that deferasirox progressively decreases iron overload over 2 years in NTDT patients, with a safety profile consistent with that of the core study.

As previously noted, LIC assessment is not always feasible and hence, alternate SF level thresholds for chelation therapy have been suggested; LIC ≥ 5 mg Fe/g dw and ≤ 3 mg Fe/g dw correspond to SF ≥ 800 ng/ml and ≤ 300 ng/ml, respectively. These SF thresholds were initially established in the THALASSA trial through correlation analysis between both iron overload indices. The ORIENT study further supports the use of SF thresholds based on their relationship with morbidity risk.

Thus, safe and effective chelation therapy is now available for NTDT patients ≥ 10 years, the age at which iron-related morbidity starts to be of concern; as well as for patients with LIC ≥ 5 mg Fe/g dw or SF ≥ 800 ng/ml, which again represent thresholds after which the risk of serious iron-related morbidity is increased.

Advances in NTDT treatment

New modalities can be divided into two categories, one that targets ineffective erythropoiesis and a second targeting iron overload in NTDT patients. Of the modalities that aim to ameliorate ineffective erythropoiesis, the two most promising are JAK2 inhibitors (TG101209) as well as Sotatercept (ACE-011) and Luspatercept (ACE-536). JAK2 inhibitors limit overproduction of immature erythroid cells in thalassemia patients, potentially reversing extramedullary hematopoiesis and preventing splenectomy. The ACE-011 and -536 products interact at late stages of erythropoiesis, eventually leading to an increase in hemoglobin production. Although promising in theory, most of the data on these molecules comes from preclinical studies and studies performed on mice. Mouse models have also suggested roles for hepcidin therapy, transferrin therapy, as well as transmembrane protease serine-6 reduction in decreasing iron overload in thalassemia patients. Human studies evaluating the role of such modalities in patients and data are eagerly awaited.

Conclusion

NTDT is a subset of thalassemia patients that do not require regular transfusion therapy for survival. It encompasses several genotypes and is mainly a clinical diagnosis. The interplay of three hallmark pathophysiologic factors, ineffective erythropoiesis, chronic anemia, and iron overload, leads to the clinical picture seen in NDTD. Therapy involves a tailored combination of Transfusions, HbF inducers, Splenectomy, and Iron chelation. Of the aforementioned, only Iron chelation therapy has clear guidelines of when to initiate, adjust, and terminate. New treatment modalities are currently being investigated as well, to broaden options available for NTDT management, with ultimate goals of prolonging longevity and improving quality of life.

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Hereditary Iron Overload

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Hereditary iron overload include several disorders characterised by the development of iron accumulation in tissues, organs, or even single cells or subcellular compartments (e.g. mitochondria in Friedreich's ataxia) [1-3].

They are determined by mutations in genes directly involved in the regulation, transport and cellular management of iron that lead to systemic or regional iron overload (Table 1).

The first ones are characterised by increased serum ferritin with or without high transferrin saturation, and with or without functional iron deficient anemia, while regional forms are characterised by normal serum iron indices or even low ferritin (e.g. neuroferritinopathy). Neuroferritinopathy belongs to a group of neurodegenerative diseases characterized by iron accumulation in the basal ganglia (Neurodegeneration with brain iron accumulation (NBIA) [4]. Friedreich ataxia, the most common recessive ataxia in the Caucasian population, is characterized by mitochondrial iron accumulation and by decreased activity of iron-sulfur cluster enzymes [5].

Table 1. Inherited diseases leading to regional or systemic iron overload due to defect of genes involved in iron homeostasis

Systemic iron overload				
Hereditary Hemochromatosis	Gene, locus	Protein; Function	Transmission, age of onset	Typical manifestations
Type 1 (HFE-HH)	<i>HFE</i> , 6p21.3	HFE; hepcidin regulation	AR, adult	Liver damage, diabetes arthropathy
Type 2 A (JH)	<i>HFE2</i> , 1q21	HJV; hepcidin regulation	AR, juvenile	Hypogonadism, heart failure, diabetes, liver damage, arthropathy
Type 2 B (JH)	<i>HAMP</i> , 19q13.1	Hepcidin; regulation of iron homeostasis (iron absorption and storage cell iron release)	AR, juvenile	Hypogonadism, heart failure, diabetes, liver damage, arthropathy
Type 3	<i>TfR2</i> , 7q22	TfR2; hepcidin regulation	AR, juvenile to adult	From HFE- to JH-like
Type 4 (A e B) (ferroportin disease)	<i>SLC40A1</i> , 2q32	SLC40A1; cellular iron export	AD, adult	Mild-moderate (4A) to moderate-severe (4B)
Other forms?	-	-	-	-

Systemic iron overload				
Hereditary Hemochromatosis	Gene, locus	Protein; Function	Transmission, age of onset	Typical manifestations
Aceruloplasminemia	<i>CP</i> , 3q24-q25	Ceruloplasmin; ferroxidase interacting with ferroportin	AR, adult	Mild microcytic anemia, Dystonia, dyskinesia, ataxia, dementia, liver damage, diabetes
Hypotrasferrinemia	<i>TF</i> , 3q22.1	Transferrin; blood iron transport	AR, infantile	Severe microcytic anemia, low s-iron, s-TF <10 mg/dL, high s-ferritin
DMT1 deficiency	<i>SLC11A2</i> , 12q13.12	DMT1; iron entry into the duodenal cell, iron exit from endosome	AR, juvenile	Microcytic anemia (moderate to severe), high TS, high s-ferritin
Regional iron overload				
Neuroferritinopathy	<i>FTL</i> , 19q13.33	L-ferritin; iron storage	AD, adult	Dystonia, spasticity, rigidity, Parkinsonism.
Friedreich's ataxia	<i>FXN</i> , 9q21.11	Frataxin; mitochondrial iron sulfur cluster biogenesis	AR, puberal	Spinocerebellar and sensory ataxia, cardiomyopathy

HH: hemochromatosis; JH: juvenile hemochromatosis; AR: autosomal recessive; AD: autosomal dominant; HJV: hemojuvelin; TFR2: transferrin receptor 2

Dysregulation of hepcidin synthesis or alterations of ferroportin function are the main physiopathologic mechanisms involved in systemic iron overload [1]. Defective hepcidin synthesis may result from either direct alteration of the *HAMP* regulatory pathway (Hemochromatosis type 1, 2, 3) or erythroid-dependent *HAMP* inhibition (Hypotransferrinemia, DMT1 deficiency) leading to increased intestinal iron absorption and release from macrophages and, in turn, to a net increase of iron influx in the body. These forms are characterised by high transferrin saturation and serum ferritin, diffuse parenchymal iron overload targeting liver, endocrine pancreas, heart, adeno-hypophysis and joints. The generation of toxic non-transferrin-bound iron (NTBI) and labile iron pool (LIP) catalyses the formation of reactive oxygen species and accumulation of lipid hydroperoxides that destroys membrane structure and function, damaging all organic molecules, eventually resulting in cell death . In Hypotransferrinemia, the very low transferrin level results in a severe functional iron-deficient anemia that, together with the lack of the transferrin-induced signal on hepatocyte surface, markedly inhibits hepcidin synthesis inducing parenchymal iron overload. Like Hypotransferrinemia, DMT1 deficiency is an extremely rare disorder characterised by functional iron deficient anemia and iron overload. Clinical observations are in agreement with a major role of DMT1 in erythropoiesis and suggest either a compensatory pathway for iron absorption or a minimal residual activity that could participate in the development of iron overload.

Ferroportin mutations lead to two distinct disorders: i. reduced ability to export iron from macrophages, enterocytes, and, to a lesser degree, from hepatocytes (Hemochromatosis type 4A), ii. reduced ability to interact with hepcidin (hepcidin resistance) as part of the negative feedback loop regulating iron absorption (Hemochromatosis type 4B). In type 4A, transferrin saturation is usually normal, and iron accumulation occurs preferentially within Kupffer cells, while in type 4B, hepcidin resistance would lead to increased iron absorption in the duodenum and to enhanced iron release from macrophages, leading to classical hemochromatosis (HH) phenotype [6].

Aceruloplasminemia is a peculiar form of systemic iron overload resulting from the lack of ceruloplasmin action in favouring cellular iron release and iron incorporation into apo-transferrin.

Mild microcytic anaemia, low serum iron, increased serum ferritin, and iron overload in the brain, pancreas, retina, and liver are typical features, leading to ataxia and dementia, diabetes, and retinal degeneration. Recently, phenotype heterogeneity has been described in Aceruloplasminemia.

Among all hereditary iron loading disorders, HH is central when considering epidemiological impact, extent of iron burden, and risk for iron-related morbidity and mortality. HFE-HH is the most frequent form and is almost exclusive of Caucasian populations. The homozygous p.Cys282Tyr genotype explains the very large majority of HFE-HH with a characteristic decreasing gradient from north to south of Europe leading to a prevalence of 0.01 in Ireland and 0.0005 or less in central-southern Italy. All the other forms of HH are rare or very rare with some clustering likely due to local founder effect. HFE-HH phenotype is extremely variable ranging from very mild to severe. Patients diagnosed in recent years have a disease characterized by milder iron overload, lower prevalence of complications as compared with those diagnosed before the discovery of HFE, and has a similar observed and expected survival. By contrast, the full-blown clinical manifestation of HH is frequent in juvenile HH (Table 1 summarizes the typical biochemical and clinical presentation of the different forms of HH). It is now clear that only a proportion of people homozygous for the p.Cys282Tyr will develop symptoms of hemochromatosis, that is the clinical appearance of iron overload and its complications. Gender, genetic background, physiological or pathological blood losses, and coexistence with other diseases, inherited or acquired, which increase or decrease iron absorption or favour the development of liver cirrhosis, might significantly modify HH phenotype [2-3]. However, only few putative genetic modifiers have been identified so far in humans and none of them was found in more than one series. Recently, an association between the rs236918 C>G variant in proprotein convertase subtilisin/kexin (PCSK7) gene and advanced liver fibrosis/cirrhosis was found in homozygous p.Cys282Tyr in a large cohort of patients from Northern Europe [7].

However, this promising result needs to be confirmed and the molecular mechanisms promoting liver fibrosis needs to be ascertained.

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Iron in metabolic syndrome

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Metabolic syndrome, defined by the presence of different metabolic alterations including overweight, altered glucose metabolism, dyslipidemia, hypertension, and non-alcoholic fatty liver disease (NAFLD), all conditions at risk for cardiovascular events, is often associated to alterations of iron parameters. More than 30% of the patients with NAFLD/metabolic syndrome, have increased values of ferritin and when liver biopsy is performed a certain amount of siderosis is detected. Identified as dysmetabolic iron overload syndrome (DIOS) (1), this condition is characterized by normal transferrin saturation, increased serum ferritin, mild liver siderosis in patients with different metabolic alterations and insulin resistance in the absence of a history of alcohol abuse. Interestingly when iron depletion was performed in patients with NAFLD, metabolic syndrome and increased ferritin, a reduction of insulin resistance was observed (2). Different genetic factors were searched for in the attempt to clear why these patients had iron overload but mutations of HFE gene, responsible for hereditary hemochromatosis, did not result significantly associated with siderosis/iron overload and thus it was advised not to look for these mutations in these patients (3).

In addition no significant relationship was detected between the presence of HFE gene mutations and severity of fibrosis. In contrast when other genetic factors were searched for, the presence of heterozygous beta thalassaemia resulted significantly associated with more severe fibrosis (4).

Interestingly when vascular damage, evaluated by the value of carotid intima media thickness and presence of plaques, was analyzed in relation to the presence of HFE gene mutations, it was found that subjects with NAFLD positive for HFE gene mutations, had milder

vascular damage suggesting that lower hepcidin as occurs in subjects positive for HFE mutations could have a protective role (5). Finally preliminary data indicate that, differently from expected, hepcidin levels in patients with NAFLD are increased suggesting a possible hepcidin resistance in these patients (6).

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Iron Chelation in Iron Loading Anemias

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The term “iron loading anemias” (ILAs) encompasses a number of heterogeneous conditions characterized by anemia and iron overload that can occur independently of red blood cells (RBCs) transfusions. A paradigm of these conditions is represented by the so-called non-transfusion dependent thalassemias (NTDT), a recently introduced category that in turn includes several hemoglobin disorders clinically milder than classic Cooley’s disease (CD), like b-thalassemia intermedia, a-thalassemia (HbH disease), HbE/b-thalassemia, HbS/b-thalassemia, and HbC/thalassemia (1). By definition, NTDT typically require transfusions only sporadically, but not for survival. Most of other ILAs are inherited and/or relatively rare, i.e. congenital dyserythropoietic anemias, and some hemolytic anemias (2). However, transfusion-independent iron overload can also occur in acquired conditions like certain subtypes of myelodysplastic syndromes (MDS), particularly in Refractory Anemia with Ring Sideroblasts (RARS) (3).

A common feature of ILAs is a substantial degree of expanded and/or ineffective erythropoiesis, which in turn is associated to increased intestinal absorption of iron (2). This is thought to be mediated by suppression of hepcidin, the liver hormone that physiologically limits the absorption of dietary iron and matches it with body iron needs (4). Indeed, with the development of sensitive hepcidin assays (5, 6), inappropriately low hepcidin levels have been demonstrated in several ILAs, including b-thalassemia intermedia (7) and RARS (8). Of note, very recently it has been shown that even in a mild condition like the b-thalassemia trait, hepcidin levels are reduced as compared to healthy individuals (9). In 2014, the long sought “erythroid regulator” of iron metabolism has been identified in mouse models of expanded erythropoiesis induced by repeated phlebotomies or injection of erythropoietin (10).

It is a member of the C1q-tumor necrosis factor-related family of proteins, which has been named “erythroferrone” (ERFE). ERFE production by erythroid precursors suppresses liver hepcidin synthesis leading to iron hyperabsorption (11). Although the above mentioned mouse model deals with acute erythropoiesis expansion, and the human analogue of ERFE has not yet formally demonstrated, it is likely that a similar mechanism underlies the development of iron overload in conditions of chronically expanded/ineffective erythropoiesis typical of ILAs (11).

Removal of iron in ILAs nearly always requires the use of chelators. At variance with iron overload secondary to chronic red blood cells transfusions (12), chelation protocols in ILAs are less well established and solid guidelines are lacking, especially in non-thalassemic and rare anemias. Of note, the correlation between serum ferritin levels and body iron stores is quite variable among different conditions, so that, for example, in b-thalassemia intermedia a clinically relevant iron overload can occur with serum ferritin levels significantly lower than in CD (7). This may be due, at least partially, to the fact that transfusional iron overload (i.e. in CD) involves primarily the macrophages, thought to be the main source of serum ferritin (13), while in NTDT iron overload occur primarily in hepatocytes. In NTDT good results have been recently reported by using deferasirox (14), which has the advantage of oral administration as compared to classical deferoxamine. A clinically relevant iron overload is not rarely observed in subjects with b-thalassemia trait in the Mediterranean area (15), and it is frequently the result of multiple factors that concur to suppress hepcidin (i.e. ineffective erythropoiesis, excess alcohol intake, co-inheritance of common hemochromatosis mutations, and others still poorly understood). This condition poses special therapeutic difficulties due to the fact that classical phlebotomies (400-450 ml each) are poorly tolerated in slightly anemic individuals, and the use of the oral chelator deferasirox is off label. We have recently reported some good results through an alternative approach consisting in “micro-phlebotomies” (150-250 ml each) (16), with or without concomitant subcutaneous deferoxamine, but further studies are required to establish the best regimens in this “orphan” condition.

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AIHA: diagnostic approach

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Autoimmune hemolytic anemia (AIHA) is a relatively uncommon and heterogeneous condition (estimated incidence 1-3 per 10⁵/year, prevalence of 17:100,000) caused by autoantibodies directed against erythrocyte self-antigens. It is usually classified according to direct antiglobulin test (DAT) positivity and thermal characteristics of the autoantibody in warm, cold, and mixed forms, although atypical cases of difficult diagnostic classification are reported with increasing frequency (1). It can be primary or secondary to lymphoproliferative syndromes, infections, immunodeficiency and tumors. Moreover, AIHA is described with increasing frequency following hematopoietic stem cell transplantation, as a complication of blood transfusion in alloimmunized patients, and in recipients of solid organ transplants when there is ABO-incompatibility between donor and recipient (passenger lymphocyte syndrome). The clinical presentation of primary AIHA may vary from mild to very severe forms, with a reported mortality of 11% in older series (1), and of ~4% in a more recent large retrospective study (2). Secondary forms are usually more severe, with an increased mortality, particularly in post-transplant forms.

The diagnosis is based on the presence of hemolytic anemia and serologic evidence of anti-erythrocyte autoantibodies by the DAT, which can be performed with various methods with different sensitivity/sensibility, level of automation, and diffusion among laboratories. The DAT-tube is the gold-standard traditional agglutination technique. It should be performed with monospecific antisera, which allow the classification of AIHAs in warm forms (DAT+ for IgG only or IgG plus C3d), cold agglutinin disease (CAD) (DAT+ for C3d only, with cold agglutinins of I specificity), and mixed forms (DAT+ for IgG and C3d, with coexistence of warm autoantibodies and high titer cold agglutinins).

Warm AIHA (~70% of all cases) are mainly due to IgG which generally react at 37°C and primarily determine extravascular hemolysis; cold forms (~20% of all cases) are due to IgM, which are able to fix complement more efficiently than other isotypes, have an optimal temperature of reaction at 4°C, and prevalently cause intravascular hemolysis. Clinically, the amount of RBC destruction by intravascular hemolysis has been calculated in 200 mL of RBCs/hr, whereas it is 10-fold lesser by extravascular hemolysis. Thus, particular attention should be given to atypical AIHA due to IgM with a thermal range close to 37°C (warm IgM) which can cause severe and fatal cases (3). These forms are also difficult to diagnose, being weakly DAT-positive for anti-C3 or DAT-negative, causing detrimental delay in therapy. These cases may be diagnosed by the Dual Direct Antiglobulin Test (4). Finally, in case of DAT positivity with polyspecific antisera and clinical features of cold-induced hemolysis, it is recommended to perform the Donath-Landsteiner test, which reveals a bithermic hemolysin able to fix complement at cold temperatures and to determine RBCs lysis at 37°C, responsible of paroxysmal cold hemoglobinuria (mainly observed as an acute form in children).

DAT with polyspecific or anti-IgG and anti-C antisera may yield false-negative results due to the presence of IgA, low-affinity autoantibodies, or numbers of RBC-bound antibodies below the threshold of the test (400 molecules per RBC). For the former two conditions, monospecific antisera against IgA and low ionic strength solutions (LISS) or cold washings should be used to overcome the DAT negativity. Small amounts of autoantibodies can be detected employing more sensitive techniques, such as microcolumn and solid-phase antiglobulin tests that are suitable for automation and nowadays available in most laboratories. These tests should be interpreted with caution, given their lower specificity compared to tube-test (5). Other more sophisticated techniques such as the complement-fixation antibody consumption test, the enzyme-linked and radiolabeled tests, the flow-cytometry, and the mitogen-stimulated (MS)-DAT may be useful to overcome DAT negativity but are not routine in the majority of laboratories.

Flow-cytometry was shown able to detect up to 30-40 molecules of anti-RBC autoantibodies, and able to allow the diagnosis with 100% sensitivity and specificity. In conclusion, despite the numerous tests available that have decreased the proportion of DAT-negative cases, in about 5% of AIHA the diagnosis is delayed after extensive laboratory investigation to exclude other causes of hemolysis, and on the basis of the clinical response to steroid therapy. In DAT-negative AIHA that require second line therapy any further non-routinely investigations should be strongly pursued to help clinical decisions. A recent large multicenter study of 308 AIHA case showed that atypical (mainly DAT-negative) have more frequently a severe onset with hemoglobin levels <6 g/dL and require multiple therapy lines, which are associated to a fatal outcome (2). It is worth reminding that that 0.01-0.1% of healthy blood donors and 0.3-8% of hospital patients have a positive DAT without clinical evidence of AIHA. Moreover the DAT may be positive after administration of various therapeutics (intravenous immunoglobulins, Rh immune globulins, antilymphocyte globulin), and in diseases with elevated serum globulins or paraproteins. Finally, the DAT is positive in conditions such as delayed hemolytic transfusion reactions caused by alloantibodies, and in the hemolytic disease of the newborn (1). Moreover, the coexistence of auto- and alloantibodies has been reported in 1/3 of AIHA patients, and their presence is often masked by autoantibodies, possibly causing severe hemolytic reactions in case of RBC transfusion. Thus, allo- and autoantibody should be differentiated by immunoabsorbance techniques and extended RBC genotyping, and antibody specificity identified in sera (indirect antiglobulin test) and eluate, particularly in cases unresponsive to blood transfusions or with unconvincing AIHA. The transfusion need may be extremely high in AIHA with reticulocytopenia (reported in 39% of children and ~20% of adults), which may be due to an anti-erythroblast reactivity, and may represent a clinical emergency. AIHA may be associated with immune thrombocytopenia (Evans syndrome), present in about 1/3 of pediatric and 1/10 of adult AIHA patients (in the latter a poor prognostic factor).

Finally, AIHA may be secondary to underlying disease: the majority of warm forms are idiopathic, whereas secondary forms are associated with various conditions, including infectious mononucleosis, systematic lupus erythematosus, autoimmune hepatitis, human immunodeficiency virus, and other lymphoproliferative or autoimmune disorders. Acute cold forms are mainly secondary to infections (Mycoplasma pneumonia, Epstein–Barr virus, cytomegalovirus), whereas chronic CAD to neoplastic or lymphoproliferative disorders. About 10% of chronic lymphocytic leukemia and 2–3% of non-Hodgkin's and Hodgkin's lymphoma develop AIHA. Moreover, a clonal bone marrow lymphoproliferation was demonstrated by bone marrow biopsy in 75% of the CAD patients, and by flow cytometry in 90% (6). Thus, an underlying lymphoproliferative disorder should be excluded by bone marrow biopsy and flow cytometry in all CAD cases prior to therapy, and in warm and mixed cases relapsed after steroid therapy.

Finally, a careful anamnesis is required to identify a possible drug-associated immune hemolysis, which is relatively rare but frequently undiagnosed. More than 150 drugs are known to be associated with immune hemolysis, which can be due to drug-independent auto-antibodies (typically methyldopa, procainamide, ibuprofen, diclofenac, fludarabine, cladribine), and drug-dependent antibodies (typically ceftriaxone, cefotetan, penicillin, piperacillin, β -lactamase inhibitors, and other antibiotics).

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Congenital Dyserythropoietic Anemias (CDAs)

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Erythropoiesis is the complex process that involves differentiation of early erythroid progenitors to mature enucleated red cells. Dyserythropoiesis is a derangement along this process and usually it causes a reduced capacity of cell production. Since this process is accompanied by anemia, it causes an increased erythropoietic signal that causes an enhanced presence of bone marrow erythroid cells.

All inherited dyserythropoiesis forms have in common bone marrow erythroid abnormality and erythroid hyperplasia appearance. The microscopic morphological evaluation justifies the heterogeneity of these syndromes. The term *dyserythropoiesis* was first used by Crookston¹ (for cases later classified as CDA II) and by Wendt and Heimpel (for cases later classified as CDA I)¹. The three classical types of CDAs have been defined on the basis of bone marrow morphology². This working classification is still used in common clinical practice³. Both CDA I and II are autosomal recessive diseases.

The hallmark of CDA I are macrocytic anemia and bone marrow appearance of incompletely divided cells with thin chromatin bridges between pairs of erythroblasts, which may also be seen between two nuclei in a single cell. On the contrary CDA II patients show a marked increase in bi- and multi-nucleated erythroblasts in their bone marrow. CDA type III is an autosomal dominant disease with giant multi-nucleated erythroblasts in bone marrow and it appears extremely rare. This latter was first reported in 1962 in a large Swedish family accounting for 34 patients.

Recently exome sequencing in the two described families allowed for the identification of *Kif23* as the causative gene⁴.

There are, however, families that fall within the general definition of the CDAs, but do not conform to any of the three classical types.

CDA type IV, a CDA II with negative serum tests, sharing similar bone marrow morphology of CDA III (multi-nucleated erythroblasts), was originally listed in the group of the CDA variants, as proposed by Wickramasinghe⁵. CDA variants with mutations in erythroid transcription factor genes (*KLF1*, *GATA-1*) have been identified³.

Up to December 2009 European registries collected 124 and 377 confirmed cases of CDA I and CDA II cases, respectively. The prevalence of both types combined varied widely between European regions, with minimal values of 0.08 cases/ million in Scandinavia and 2.60 cases/million in Italy. CDA II is more frequent than CDA I, with an overall ratio of approximately 3.2, but the ratio also varied between different regions. The most likely explanations for the differences are both differences in the availability of advanced diagnostic measures and different levels of the awareness for the diagnosis of the CDAs. The estimations reported are most probably below the true prevalence rates, due to failure to make the correct diagnosis and to underreporting.

The gene responsible for CDA I (*CDANI* gene) was mapped to the long arm of chromosome 15 between 15q15.1q15.3 by homozygosity mapping performed. The *CDANI* gene was successively cloned with 28 exons spanning 15 Kb and encoding a protein named codanin-1⁶. In unrelated patients of European, Bedouin and Asian origin, different point mutations were detected. Approximately 90% of patients with a bone marrow suggesting CDA I have codanin-1 gene mutations.

All these seem to be independent events and up to now no particularly frequent mutations are present in literature data. Interestingly, no homozygotes or compound heterozygotes for null-type mutations have been identified, supporting an earlier notion that codanin-1 may have a unique function and may be essential during development. Codanin-1 is a ubiquitously expressed protein, still not well characterized. It seems to be related to chromosome structure and it must be involved in mitotic process⁷.

Recently several families *CDANI* mutation negative allowed for the identification of a new causative gene: C15ORF41, coding for a protein involved in DNA replication and chromatin assembly⁸.

Sequencing analysis of CDA II patients showed a wide spectrum of different mutations in SEC23B gene in either the compound heterozygous or homozygous state. The disease gene encodes the cytoplasmic coat protein (COP)II component SEC23B, involved in the secretory pathway of eukaryotic cells⁹. COPII is a multi-subunit complex which mediates accumulation of secretory cargo, deformation of the membrane and generation of subsequent anterograde transport of correctly folded cargo that bud from the ER towards the Golgi apparatus. The specificity of the CDA II phenotype seems to be determined by tissue-specific expression of SEC23B versus SEC23A during erythroid differentiation.

An attempt to identify a genotype-phenotype relationship demonstrated that patients with compound heterozygosity for a missense and nonsense mutation tended to produce more severe clinical presentations than homozygosity or compound heterozygosity for two missense mutations¹⁰. Homozygosity or compound heterozygosity for two nonsense mutations was never found, and it was supposed to be lethal. Very recently *Sec23b* deficient mice was generated and it has not apparent anemia phenotype, but die shortly after birth, with degeneration of professional secretory tissues, pancreas as well as salivary glands. These data demonstrate that *Sec23b* deficient humans and mice exhibit disparate phenotypes, apparently restricted to CDA II in humans and a prominent neonatal pancreatic insufficiency in mice.

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Disorders of Neurosiderosis

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Iron is the most abundant transition metal in biology and an essential cofactor for many cellular enzymes. In particular, in brain iron is vital for the activity of various enzymes implicates in the neurotransmitter synthesis and catabolism and thus it is indispensable for the normal development of brain function. However, iron is also highly toxic, due to its involvement in reactions that lead to hydroxyl radical species formation. For these reasons, systemic iron homeostasis is tightly regulated and its perturbation is an initial cause of several disorders, including genetic and sporadic neurodegenerative disorders. As recently revealed by MRI techniques, iron is abnormally deposited in brains of subjects affected by several neurodegenerative disorders, including the most frequent ones, such as Parkinson and Alzheimer Diseases, where the metal is accumulated in specific regions that undergo neurodegeneration (Ward et al., 2014). An opportunity to investigate the iron involvement in neurodegenerative processes is the group of diseases, referred to as “Neurodegeneration with Brain Iron Accumulation”, in which diagnostic imaging and autopsy findings reveal focal iron accumulation in specific regions of the brain, usually in the basal ganglia (Levi and Finazzi, 2014). They are a set of degenerative extrapyramidal monogenic disorders characterized by early or late onset, with the main symptoms associated to problems in the movement, spasticity and cognitive impairment. Up to now, 10 different genetic forms have been identified as causative of NBIA, but this number is likely to increase in short time. Two forms are linked to mutations in genes directly involved in iron metabolism: Neuroferritinopathy (NF) and Aceruloplasminaemia, associated to mutations in the *FTL1* and ceruloplasmin genes, respectively. In the other forms the connection with iron metabolism is not evident at all and the genetic data suggest the involvement of other pathways.

The affected *PANK2*, *COASY*, *Pla2G6*, *C19orf12*, and *FA2H* genes seem to be related to lipid metabolism and to mitochondria functioning, *WDR45* and *ATP13A2* genes are implicated in lysosomal and autophagosome activity, while the *C2orf37* gene encodes a nucleolar protein of unknown function (Levi and Finazzi, 2014). The *in vitro* and *in vivo* studies on these disorders begin to elucidate common features that may point to share pathogenic pathways among them. Aceruloplasminemia and NF have in common iron mis-handling and the consequent cellular oxidative damage, while the involvement of lipid metabolism and mitochondria is evident in many of the other diseases. Mitochondria iron accumulation is a feature often observed in disorders with defective iron sulfur cluster assembly, such as Friedrich Ataxia or X-linked sideroblastic anemia with ataxia (Levi and Rovida, 2009). Interestingly, mitochondrial iron overload often cause cytosolic iron deprivation and promote a vicious cycle with increased iron uptake and subsequent deposition in mitochondria (Isaya, 2014). Furthermore, the perturbation of the synthesis/remodeling of membrane lipids might interfere with mitochondria functionality, lysosomal activity and in the clearance of damaged mitochondria (mitophagy).

Our data on two of these disorders, NF (Maccarinelli et al., 2014) and Pantothenate Kinase-2 associated Neurodegeneration (Santambrogio et al., 2015), confirm the participation of all these elements to the pathogenetic mechanisms, suggesting that the interplay among membrane homeostasis, mitochondria functioning and iron metabolism play a fundamental role in the development of different types of NBIA.

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