Chapter

The Rare Anaemias

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Abstract

Anaemia is a common worldwide disorder mainly due to iron or vitamins deficiency. However, among the rare diseases (RD), there is a group associated with anaemia as main clinical manifestation or rare anaemias (RA). RA are mostly hereditary, and since they are little known, even for professionals, they remain undiagnosed, or misdiagnosed, for very long periods of time. This creates in patients, or, in their parents (if they are children) a permanent situation of stress due to the absence of a diagnosis, the impossibility to predict the course of the disease, and to the impossibility to perform, genetic counselling for future pregnancies. About 83 different RA have been described and their mechanism is in general a bone marrow or a red blood cell (RBC) defect. The most well-known RA are Fanconi anaemia, the thalassemia syndromes, sickle cell disease, hereditary haemolytic anaemias and paroxysmal nocturnal haemoglobinuria (PNH). The main objective of this chapter is to offer a review of the state of the art of RA knowledge and a way to facilitate their identification and final diagnosis through clinical manifestations and laboratory diagnostic tests.

Keywords: anaemia, red blood cell erythropoiesis, haemoglobin, haemolysis, haemoglobins, enzymes, membrane

1. Introduction

Anaemia is very common condition in human pathology that may result from a wide variety of causes, either congenital or acquired. It is always the manifestation of an underlying disease, and never a disease by itself. Haemoglobin concentration (Hb) is the most reliable indicator of anaemia, but since its normal distribution at population level varies with age, sex, and physiological status, the Word Health Organization (WHO) has defined the existence of anaemia when Hb is less than 110 g/L in children and pregnant women, 120 g/L in non-pregnant women and 130 g/L in men. Moreover, measuring Hb is relatively easy and inexpensive, and currently, all automated and semi-automated haematology analysers measure Hb with great precision and accuracy. It is well known that iron deficiency in children and women and chronic diseases in adults and elderly, is the most frequent cause of mild to moderate anaemia worldwide [1, 2].

In general, there are three primary causes of anaemia: (1) Bone marrow erythropoietic defects associated with or without reduced haemoglobin synthesis. (2) Haemolysis or excessive destruction of mature red blood cells (RBCs), and (3) blood loss or bleeding. Anaemia can be the consequence of a single disease (e.g. haemoglobinopathy, enzyme deficiency, etc.), but it can be also the expression of external factors such as nutritional deficiencies, parasitic or viral infection, and other. However, there is a group of anaemias that is considered rare because their frequency in our population is less than 5 cases for 10,000 individuals. These are the

so-called rare anaemias (RA), mostly of congenital origin [3]. RA are an important, and relatively homogeneous group of rare diseases (RD), where anaemia is the first and most relevant clinical manifestation of the disease. This importance was recognized, for first time, by the European Commission (EC) that in 2002 approved the co-financing of the DG-SANCO Project: "European Network for rare and congenital diseases" (ENERCA; www.enerca.org). Interestingly, this Project started shortly before the creation of the High Level Group (HLG) in 2004 that brought together experts from all the Member States (MS) in several areas of RD expertise. For RA, this was a great advantage, because it facilitated the progressive development of ENERCA Project in parallel to the development of the different HLG areas of action: (a) Patient safety and quality of care, (b) Health impact assessment and health systems, (c) Health technology assessment, (d) European workforce for health professionals, e) European reference networks, (f) Information and e-health and more recently, (g) Cross-border healthcare purchasing and provision [4].

Before ENERCA, RA were almost unknown in Europe, including some health professionals, because in many cases, the cause of the anaemia was not known and/or there is no treatment available. Moreover, for many years, anaemias, in general, have been underestimated by public health providers, due to its frequent misdiagnosis with iron deficiency anaemia, the most frequent cause of anaemia worldwide. ENERCA changed definitively this situation by developing three consecutive phases with a total duration of 15 years (www.enerca.org). The first ENERCA Project (ENERCA 1), starting in 2003, dedicated to congenital RA, only, allowed the establishment of the necessary background for a sustainable coordination in the area of health information, collection of epidemiological data, comparability issues, exchange of data and information within and between MS. At this time, it also facilitated a rapid reaction to RA diagnosis and treatment. The second ENERCA Project (ENERCA 2) starting in 2005, covered, in addition, to congenital anaemias, other rare causes of anaemia, either hereditary or acquired, and dedicated more and stronger activities to health Information, patient's data collection, education and training and quality assessment for special RA diagnostic procedures. This has provided a first and unique approach for prevention, diagnosis and treatment of RA in the word. The third ENERCA Project (ENERCA 3), starting in 2009, was co-financed by the EC through its Executive Agency for Health and Consumers (EAHC) and its objectives were parallel to the strategic objectives of the EU Health Programme 2008–2013 consistent in the creation of a *European Reference Network (ERN) of Centres of Expertise (CEs) in Rare Anaemias* (**Figure 1**).

After 2013, the EC has approved co-financing ENERCA Project for an additional 3 years period (2014–2016), with the aim of developing and implementing the new e-health information and communication technologies (ICT) for assuring the same access to health services in RAs across Europe, independently from the place of residence. This new and last, but not least, ENERCA Project, called e-ENERCA, was based, in part, on previous ENERCA projects achievements, but adapted to the Directive 2011/24/EU of 9 March 2011 on the *application of patients' rights in cross-border healthcare has* become a very important tool for the promotion of Rare Diseases (RD) European Reference Networks (ERN) that, within that general framework, have provided the following benefits:

- Access to experts and expertise throughout the European MS for both patients and health professionals, independently of the country of origin or practice, reduce inequalities and maximize the cost-effective use of resources;

- Epidemiological surveillance throughout EU by gathering comparable data on patients affected by RAs and allowing the implementation of preventive programmes for tackling RAs;

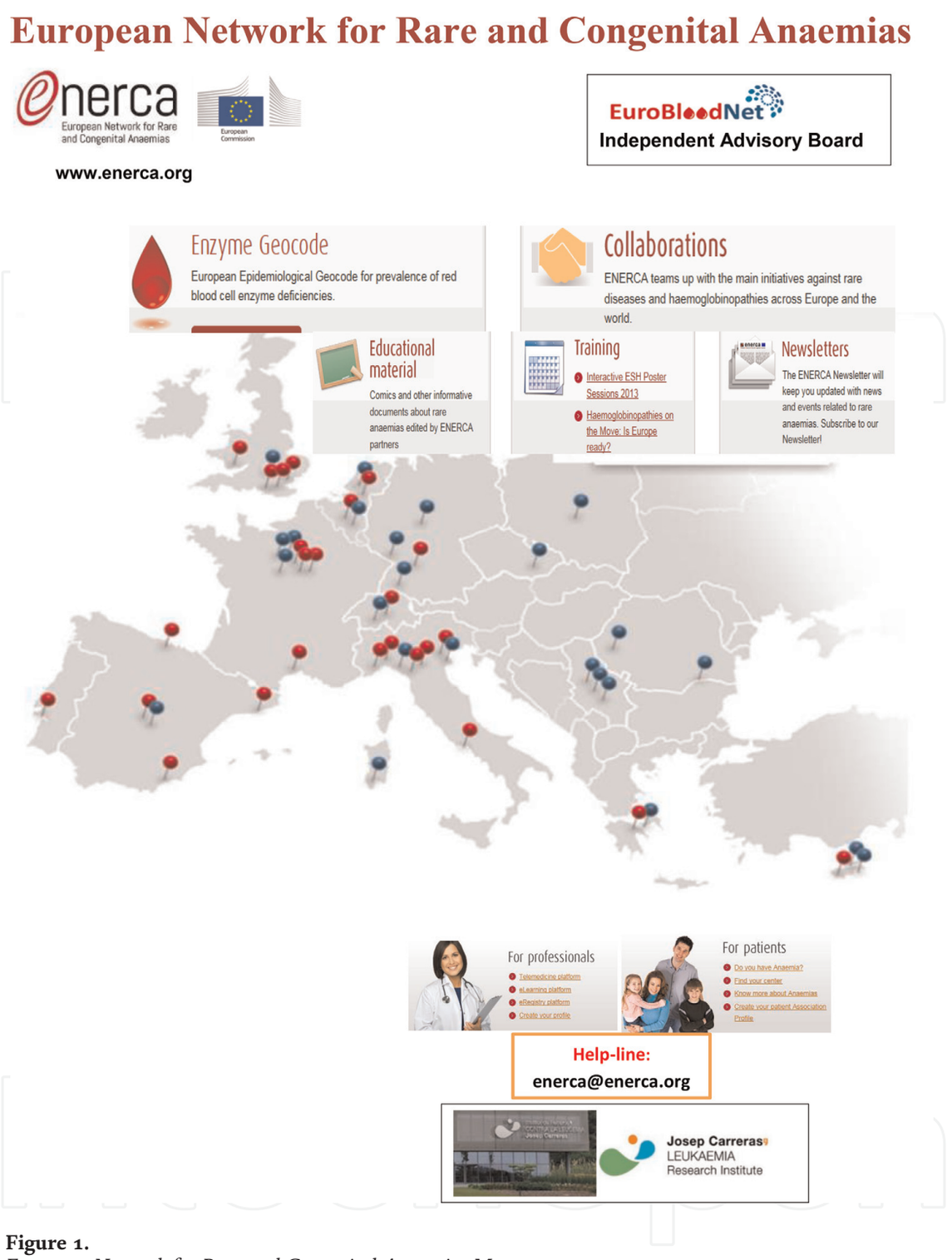

Figure 1.
European Network for Rare and Congenital Anaemias Map.

- Fostering of best practices for prevention, diagnosis and clinical management;
- Promotion of knowledge dissemination of share of expertise and support of research, and increase awareness about RAs;
- Facilitate the transposition of the Directive 2011/24/EU of 9 March 2011 on the application of patients' rights in cross-border healthcare.

For maintaining its sustainability, in March 2016, ENERCA applied for the ERN European Commission (EC) Call, by expanding the rare anaemias to all the other rare haematological diseases (RHD), including oncological and non-oncological diseases [5].

Currently, the ERN for Rare Haematological Diseases (RHD), called EuroBloodNet (www.eurobloodnet.eu), is one of the 24 recognized ERNs, and after its second year of life, can be considered an acceptable tool for the improvement RHD diagnosis and for the provision of high-quality healthcare to all patients who have conditions requiring a particular concentration of resources or expertise. In brief, EuroBloodNet will also provide focal points for medical training and research, information dissemination and evaluation, and will contribute to the establishment of national contact points for RHD. Up to now, however, only 66 Centres in Europe are recognized as Health Care Providers (HCP) for RHD, a situation created by the extremely different endorsement decisions of individual Members States (MS). So, we have arrived to the astonishing situation in that many of the ENERCA experts, well recognized by the state of the art and ENERCA White Book recommendations, are not included as EuroBloodNet experts because they do not belong to an national recognized Healthcare Provider (HCP) and cannot take participate in the Network activities and/or take profit from their advantages. In order to overcome this restraint, a new EC call for membership application to the existing European Reference Networks (ERNs) has been launched on September 30, 2019.

Hereditary (>80%)
Erythropoietic defects (non-regenerative anaemias)
• Fanconi anaemia (FA) • Diamond-Blackfan anaemia (DBA) • Congenital dyserytropoietic anaemia (CDA)
RBC defects (regenerative anaemias)

• Thalassaemia syndromes (Cooley anaemia)
• Sickle-cell disease (SCD)
• Hereditary membranopathies
  ◦ Hereditary spherocytosis (HS)
  ◦ Hereditary elliptocytosis (HE)
  ◦ Hereditary stomatocytosis (HSt)
• Erythroenzymopathies
  ◦ Glucose-6-phosphate dehydrogenase deficiency (Favism)
  ◦ Pyruvate kinase deficiency (PKD)
  ◦ Ultra-rare erythroenzymopathies associated with or without muscular or neurological disease

Iron metabolism defects (non-regenerative anaemias)
• Congenital sideroblastic anaemia (CSA) • Non-sideroblastic anaemias with microcytosis (IRIDA)*
Acquired (<20%)
Erythropoietic defects (non-regenerative anaemias)
• Bone marrow aplasia (BMA) • Pure red cell aplasia (PRCA) • Myelodysplastic syndromes (MDS)
RBC defects (regenerative anaemia)
• Paroxysmal nocturnal hemogobinuria (HPN)
Blood plasma abnormalities (regenerative anaemias)
• Autoimmune haemolytic anaemia (AIHA)
Microcirculation defects (regenerative anaemias)
• Haemolytic uremic syndrome (HUS)
*Iron refractory iron deficiency anaemia.

Table 1.
General classification of rare anaemias.

2. Classification

The RA are classified into two main groups: Hereditary and acquired, and in these groups, the mechanism of the anaemia can be sub classified into five different defects: (1) bone marrow (erythropoietic), (2) peripheral blood (red blood cell), (3) iron metabolism (sideroblastic and non-sideroblastic), (4) blood plasma (autoimmune haemolytic anaemia and related syndromes) and (5) microcirculation (haemolytic uremic syndrome and other microangiopathic disorders). This means that all RA are the consequence of haematopoietic system defects leading to a low RBC production (erythropoietic defects), or of RBC defects, either intrinsic or associated to plasma or microcirculation disorders, leading to low RBC survival or haemolysis (**Table 1**).

ENERCA is actively contributing to the WHO Update Platform ICD-10 of blood and blood forming organs. In this platform, anaemias, are classified into three main groups: D50-D53 (nutritional anaemias), D54-D59 (haemolytic anaemias), and D60-D64 (aplastic and other anaemias). This ICD classification includes all kind of anaemias, hereditary, acquired, common and rare, and ENERCA has extracted the RA group that has been individually listed in the *ENERCA Web page (www.enerca.org).*

For practical purposes, according to their mechanism, prevalence and/or relevant clinical and/or social impact in the European population, ENERCA has classified the Rare Anaemias into 10 different groups [6]:

Group 1. Haemoglobin disorders: Haemoglobinopathies and Thalassaemias.

Group 2. Hereditary Haemolytic Anaemias (HHA): Red blood cell enzymopathies and membrane defects.

Group 3. Hereditary erythropoietic failure or aplasia: Diamond-Blackfan anaemia (DBA) and Fanconi anaemia (FA),

Group 4. Congenital dyserythropoietic anaemias (CDA),

Group 5. Hereditary sideroblastic anaemias,

Group 6. Hereditary non-sideroblastic anaemias due to iron metabolism defects,

Group 7: Hereditary disorders of folic acid and cobalamin defects.

Group 8: Paroxysmal nocturnal haemoglobinuria (PNH),

Group 9: Anaemias due to rare complex mechanisms and.

Group 10: Anaemias of unknown origin (AUO).

The underlying cause of rare anaemias remains still unexplained in about 20% of patients, almost one third of which might be accounted for myelodysplastic syndromes. AUO can be also due to complex clinical situations and multifactorial mechanisms, in general associated with systemic, non-haematological, hereditary or acquired diseases. Their existence is a very important tackling exercise for clinical and biological research.

3. Diagnostic approach

More than 80% of RA are hereditary and, therefore, have no curative treatment, exception made of palliative therapies such as blood transfusions or erythropoietic stimulating drugs (Erythropoietin). In clinical practice they may be some confusion between RA and the anaemias that appear in the course of non-haematological or systemic diseases, also called secondary anaemias. This confusion is due to the fact that anaemia is not a disease, but a clinical manifestation, and some Rare Diseases (RD) are associated with anaemia, moderate or severe. One example of this is the Rendu-Osler disease (hereditary telangiectasia), a relatively well known RD where anaemia, due to iron deficiency, is very common, and sometimes the first clinical manifestation of the disease. Furthermore, the anaemias due to rare chronic inflammatory diseases, vitamin deficiencies, immune diseases, malignancy, or other

rare disorders, may probably be also considered RA, although they have not yet been included in this group.

Hereditary RA, as in other RD, the low number of patients creates the need to mobilize resources and their study can be efficient only if done in a coordinated European scene of action level. Among hereditary anaemias, haemoglobinopathies are the commonest genetic defect worldwide with an estimated 269 million carriers [7]. They are the consequence of mutations in the globin genes, which are responsible for the synthesis of haemoglobin, the main component of RBCs. These mutations are leading to abnormal proteins (haemoglobin variants) or to a decreased synthesis of globin chains (thalassaemias). In Europe, certain populations are particularly at risk of having a haemoglobinopathy. In Southern countries, their prevalence is higher than in central or northern Europe, but in all cases the prevalence is less than 1 per 2000 individuals. For this reason, in Europe, haemoglobinopathies and thalassaemias are considered a particular group of RD or RA. Whereas thalassaemia syndromes are inherent in the autochthonous European at risk groups (Mediterranean anaemia), other haemoglobinopathies have been imported by migration (Sickle-cell anaemia).

In general, the diagnosis of a RA is often prompted by pallor, noticed by the patient, the family, and/or the General Practitioner (GP). Severity of clinical manifestations is directly proportional to the acuteness of onset, and many patients do not notice any symptoms when anaemia occurs insidiously. At the laboratory level, the diagnosis of anaemia includes two main steps:

3.1 General diagnostic tests

General diagnostic tests always include a Complete Blood Count (CBC), the reticulocyte count and the peripheral blood cell morphology examination. The CBC includes four main parameters: (a) haemoglobin concentration (Hb), the key for anaemia diagnosis, (b) RBC count or concentration of RBCs, given as number of cells per litre of blood, (c) haematocrit or packed cell volume (PCV), given as the percentage of blood by volume that is occupied by the RBCs and (d) RBC indices or calculations derived from (a), (b), and (c), of great help for the diagnosis and classification of anaemias. These indices are automatically measured by modern haematology full automated or semi-automated, analysers, and are mainly three: (1) the mean corpuscular volume (MCV) or average size of the RBCs expressed in femtolitres (fl), (2) the mean corpuscular haemoglobin (MCH) or average amount of haemoglobin inside a single RBC expressed in picograms (pg) and (3) the mean corpuscular haemoglobin concentration (MCHC) or average concentration of haemoglobin in the RBC expressed as a percent. Sometimes the RBC distribution width (RDW), a measure of the variation of RBC size, can be also used for anaemia classification. Usually RBCs have a standard size of about 6–8 μm, but in certain disorders, a significant variation in RBC size can be present. Here the RDW value is a relatively good indicator of RBC size heterogeneity. RDW is especially useful to differentiate iron deficiency (increased value) from thalassemia (normal value). Reticulocyte count or number of circulating young RBCs (reticulocytes) is an important complementary test which indicates the bone marrow capacity to overcome the severity of anaemia [8]. Accordingly, anaemias due to RBC destruction (haemolysis) are characterized by increased reticulocyte count (*regenerative* anaemias), whereas anaemias due to erythropoietic insufficiency (aplasia or dyserythropoiesis) are characterized by a lower than expected reticulocyte count from the severity of the anaemia (*Non-regenerative* anaemias). In thalassaemias, where erythropoietic insufficiency coexists with some degree of haemolysis, the reticulocyte count may be variable.

The reticulocyte count and MCV are, up to now, the most useful criteria for anaemia classification. According to MCV, anaemias are classified into microcytic

(low MCV), macrocytic (high MCV) and normocytic (normal MCV). The two main causes of microcytic anaemias are iron deficiency and thalassaemia and the two main causes of macrocytic anaemias are cobalamin (vitamin B12) and folic acid deficiencies. Normocytic anaemias can be due to several different causes, not related with nutritional defects or thalassaemia, being the most frequent haemolysis and erythropoietic failure. Here, the reticulocyte count is the most useful test to differentiate these two conditions. In clinical practice, the most frequent cause of anaemia is iron deficiency (ID), characterized by a low MCV (microcytic anaemia). In southern Europe countries with higher "at risk" thalassaemia population (Mediterranean basin), this hereditary disorder can be misdiagnosed as iron deficiency anaemia (IDA) because of the low MCV (<82 fL) or microcytosis. Accordingly, in a patient with microcytosis the first step is always to exclude ID. If present, iron supplementation has to be given until the MCV recovers its normal value. However, if after treatment, the MCV remains low, the coexistence of a thalassemic gene has to be investigated. It should be mentioned that there are a number of conditions where the MCV can falsely rose masking the main clue of thalassaemia diagnosis. This is the case in some patients with thalassaemia who co-inherit another cause of haemolytic anaemia leading to an increased reticulocyte count. This can falsely increase the value of MCV and masking the diagnosis of thalassemia if only the MCV is used for initial screening.

As part of the CBC, the blood film examination is sometimes very useful because it may provide a clue to the diagnosis of a particular RBC defect [9]. Despite the advances in automated blood cell counting, the blood film examination retains a crucial role in the diagnosis of RBC disorders. This is particularly important in haemolytic anaemias and in the differential diagnosis of macrocytic anaemias. RBC morphology examination provides in some cases (e.g. red blood cell membrane disorders, sickle-cell anaemia), a definitive diagnosis, but, more often, it suggests a differential diagnosis that indicates further study. Morphological changes such as basophilic stippling and target cells in the blood film are not definitively associated with a haemoglobinopathy, but would be helpful findings in patients with moderate or severe anaemia associated with low MCV (Thalassaemia intermedia, or Thalassaemia major). Finally, RBC morphology examination has also the advantage of speed that may be important in severe anaemias such as those mentioned before.

3.2 Cause-oriented specific diagnostic tests

These tests are the next step for the identification of the cause of the anaemia or of its mechanism. They include a group of laboratory procedures depending on clinical or laboratory diagnostic orientation of the anaemia [8] and, when necessary, a final genetic identification of the cause of the disease [10]. In order to provide a first approach to the cause of the anaemia, several diagnosis oriented flowcharts can be found in the literature, mainly based on the morphological classification of the anaemia (microcytic, macrocytic and normocytic).

ENERCA website (www.enerca.org) provides practical flowcharts for the diagnostic orientation of anaemia. For this, three patient's data have to be provided: sex, Hb and MCV. If anaemia is detected, one of the three available flowcharts will appear, depending on the MCV value: low (microcytic anaemia), high (macrocytic anaemia) and normal (normocytic anaemia). These flowcharts are not exhaustive and the final diagnosis always requires the advice of a health professional, but they provide the basic information on how the investigation of anaemia causes can be undertaken in routine clinical practice. Using these flowcharts the most frequent RAs (haemoglobinopathies, thalassaemias and haemolytic anaemias) can be easily recognized. Depending on the results of the recommended basic tests, more specific tests (including molecular biology) can be performed. Some of these specific tests

can also be performed in general haematology laboratories but other tests require to be undertaken in specialized laboratories. In all the cases External Quality Assessment Schemes (EQAS) are necessary for assessing the quality of practice or for obtaining a technical qualification. Since the most specific tests are performed in few specialized laboratories, local (national or regional) EQAS organizations cannot establish a specific EQAS for these procedures due to its high cost. Accordingly, the EQAS for these procedures have to be promoted at European level as ENERCA 3 has done with some rare diagnostic tests for RA such as Pyruvate-kinase deficiency (PKD).

4. Rare Anaemias due to bone marrow defects

Bone marrow failure syndromes (BMFS) are multisystem diseases that are characterized by varying degrees of deficiency in the production of haematopoietic cells, which can range from the depletion of a single cell lineage (cytopenia) to that of multiple lineages or even of all lineages (pancytopenia). The most well-known acquired BMFS is aplastic anaemia (AA).This causes a deficiency of all three blood cell types (pancytopenia): red blood cells (anaemia), white blood cells (leukopenia), and platelets (thrombocytopenia) and aplastic refers to the inability of stem cells to generate mature blood cells. It is more frequent in people in their teens and twenties, but is also common among the elderly [11]. It can be caused by heredity, immune disease, or exposure to chemicals, drugs, or radiation. However, in about half the cases, the cause is unknown. The definitive diagnosis is by bone marrow biopsy; normal bone marrow has 30–70% blood stem cells, but in aplastic anaemia, these cells are mostly gone and replaced by fat. First line treatment for aplastic anaemia consists of immunosuppressive drugs, typically either anti-lymphocyte globulin or anti-thymocyte globulin, combined with corticosteroids and cyclosporine. Haematopoietic stem cell transplantation is also used, especially for patients under 30 years of age with a related matched marrow donor [11]. Congenital BMFS are, as AA, multisystem diseases characterized by varying degrees of deficiency in the production of haematopoietic cells, which can range from the depletion of a single cell lineage (cytopenia) to that of multiple lineages or even of all lineages (pancytopenia). In general they are monogenic diseases with high genetic heterogeneity and phenotypic overlapping, so a bone marrow and genetic analysis is required to reach a correct diagnosis [12]. They are ultra-rare diseases with a usual presentation during childhood and with an incidence of one to two cases per one million individuals. In almost all cases they are associated with morbidity and mortality, requiring lifelong blood transfusions, treatment of infections, growth factors and transplantation of haematopoietic progenitors. Likewise, they present a high risk of developing haematologic cancer or solid tumours and a high toxicity to treatment, which leads to a lower life expectancy. The most relevant aspects of some of these syndromes are the following:

4.1 Fanconi anaemia (FA)

FA (OMIM 227650) is a hereditary disease with a predominantly autosomal recessive pattern and in one case linked to chromosome X. Its prevalence in the general population is estimated at two to five cases per one million individuals, with an estimated incidence of 1/131,000 births. 21 different gene mutations have been identified so far to be a cause of congenital aplasia [13]. In most cases, each of the parents carries one of the pathogenic variants, with three exceptions: male patients of the FA-B subtype (FANCB gene), patients of the FA-R subtype (RAD51 gene),

and the cases in which one of the two variants is de novo (not present in the parents or present in only some of the gametes of one of the parents). The gene FANCB is a gene linked to the X chromosome, so that women are asymptomatic carriers of the pathogenic variant. From the clinical point of view, FA is considered as a syndrome of chromosomal instability with a high spectrum of clinical manifestations that can be grouped into congenital malformations, endocrine dysfunction, haematological abnormalities such as severe cytopenias, myelodysplastic syndrome (MDS) or acute myeloid leukaemia (AML) Moreover, a predisposition to develop tumours and chromosomal fragility has been noted and in about 30% of the patients do not present congenital malformations and are only diagnosed at the time when the disease debuts with the haematological abnormalities [13]. To make a diagnosis, it is necessary to confirm the chromosomal fragility by cytogenetics [14] and until a few years ago, genetic testing was not part of the routine clinical analysis of AF patients, partly because it was considered sufficient with the cytogenetic confirmation of the diagnosis (chromosomal fragility) and the subtype of the patient, and on the other hand, because the mutational analysis was boring. However, with the implementation of new high-throughput sequencing technologies [15], subtyping and mutational study are now achieved at the same time, sequencing all AF genes in the same assay, and even identifying new genes involved (**Table 2**). However, this approach

FA Subtype	FA mutated Gen	Chromosome location	Protein
A	*FANCA*	16q24.3	FANCA
B	*FANCB*	Xp22.31	FANCB
C	*FANCC*	9q22.3	FANCC
D1	*FANCD1/BRCA2*[*]	13q12.13	BRCA2
D2	*FANCD2*	3p25.3	FANCD2
E	*FANCE*	6p21–22	FANCE
F	*FANCF*	11p15	FANCF
G	*FANCG*	9p13	FANCG
I	*FANCI*	15q25-26	FANCI
J	*FANCJ/BRIP1*[*]	17q22-24	BRIP1
L	*FANCL*	2p16.1	FANCL
M	*FANCM*	14q21.3	FANCM
N	*FANCN/PALB2*[*]	16p12.2	PALB2
O	*FANCO/RAD51C*[*]	17q25.1	RAD51C
P	*FANCP/SLX4*	16p13.3	SLX4
Q	*FANCQ/ERCC4*	16q24.3	XPF
R	*FANCR/RAD51*	15q15.1	RAD51
S	*FANCS/BRCA1*	17q21.31	BRCA1
T	*FANCT/UBE2T*	7q36.1	UBE2T
U	*FANCU/XRCC2*	7q36.1	XRCC2
V	*FANCV/REV7*	1p36.22	MAD2L2
W	*FANCW/RFWD3*	16q23.1	FANCW

[*] *Gene defects that predispose to the development of cancer in FA carriers.*

Table 2.
Genetic subtypes and genes which mutation are implicated in the development of Fanconi anaemia.

can cause errors in these patients, due to the broad mutational spectrum of the disease and because all the genes related to the disease have not yet been described. For this reason, it is advisable to always start the diagnosis by the mutational study in patients with a positive chromosomal fragility for AF. With regard to treatment, in addition to correcting, as far as possible, some of the congenital malformations, it is essential to carry out a haematological follow-up in order to identify early signs and symptoms of bone marrow (BM) failure. Current guidelines recommend tracking blood counts every three to four months, and an annual bone marrow aspirate. Treatment should be initiated depending on the patient's clinical commitment and continue with it in accordance with the response to it. Support measures include transfusions of red blood cells or platelet concentrates, or the use of colony / cytokine stimulating factors. Currently, the only curative treatment for bone marrow failure in these patients is the allogeneic haematopoietic stem cell transplant (HSCT) from suitable donors. Finally, the correct and early diagnosis of AF does not only allow the discarding of other diseases, but fundamentally enables the proper management of their haematological alterations and genetic counselling to the individual and his family in case of successive pregnancies. In addition, in families with mutations in genes predisposing to cancer, it allows adequate monitoring and risk assessment in family members.

4.2 Diamond-Blackfan anaemia (DBA)

DBA (OMIM 105650), is a rare disease with a dominant autosomal inheritance pattern. No differences were observed between men and women (ratio 1:1) and the short stature is the most frequent anomaly followed by alterations in the thumb. Other less frequent anomalies have been described, such as craniofacial alterations, cleft lip, cleft palate and short neck. In DBA, the first description was a pure neonatal anaemia that required transfusion support. At present, about 1000 cases have already been described in the literature, and approximately 25% of them have at least one congenital anomaly. The average age for diagnosis is 3 months, ranging from birth to 64 years of age, with 98% of cases diagnosed in the first year of life. DBA has its molecular basis in heterozygous mutations in the genes encoding the components of either the small 40S or the large 60S ribosomal subunits that affect the processing of ribosomal RNA. Recently, DBA has also been related to mutations in the GATA-1

DBS Subtypes	% of patients	*Locus*	Mutated Gene	Protein
AD	25	19q13.2	*RPS19*	RPS19
AD	2	10q22–23	*RPS24*	RPS24
AD	1	15q25.2	*RPS17*	RPS17
AD	7	1p22.1	*RPL5*	RPL5
AD	5	1p36.11	*RPL11*	RPL11
AD	3	3q29	*RPL35A*	RPL35A
AD	1	2p25.3	*RPS7*	RPS7
AD	7	6p21.31	*RPS10*	RPS10
AD	3	12q13	*RPS26*	RPS26
AD	<1	17p13.1	*RPL26*	RPL26
AD	<1	14q21.3	*RPS29*	RPS29
AD	<1	18q21.1	*RPL17*	RPL17
AD	<1	3p24.2	*RPL15*	RPL15

DBS Subtypes	% of patients	*Locus*	Mutated Gene	Protein
AD	<1	17p13.1	*RPL26*	RPL26
AD	<1	17q12	*RPL19*	RPL19
AD	<1	2q11.2	*RPL31*	RPL31
AD	<1	8q12.1	*RPS20*	RPS20
AD	<1	19p13.2	*RPS28*	RPS28
LX	<1	Xp11.23	*GATA-1*	GATA1
Unkown	40	—	—	—

Source: [35].

Table 3.
Genetic subtypes and genes implicated in Diamond-Blackfan anaemia (DBA).

transcription factor [16], but about 45% of patients with ADB lack mutations in these identified genes, as shown in **Table 3**. It is likely that the genetic defects in this pathology accelerate the apoptosis of erythroid progenitor cells, since it has been shown that MDM2, a central regulator of p53, that acts as a ubiquitin ligase that leads to the degradation of p53, binds specifically to several free ribosomal proteins. From the clinical point of view, the diagnosis of DBA is complex. Blood counts generally show a macrocytic anaemia with reticulocytopenia, as well as elevated Hb F and RBC adenosine deaminase (ADA) in more than 85% of patients. In the bone marrow, a pure erythroblastopenia without abnormalities in the other haematopoietic cell lines is characteristic. The cumulative incidence for MDS, AML, and some solid tumours (osteosarcoma, Hodgkin's lymphoma) has been estimated at 20% at 46 years, which implies an increased risk of developing cancer. The treatment is based on the response of a large number of patients to steroids. However, it is advised not to perform this treatment until after the first year of age. Until then, and in those patients who do not respond to steroids, or who require them at very high doses, the only treatment will be adequate transfusion support with packed red blood cells. The only curative treatment for the disease is the HSCT, provided the existence of an identical HLA family donor [11].

4.3 Congenital dyserythropoetic anaemia (CDA)

CDA is, in fact, a heterogeneous group of defects of erythropoiesis with medullary abortion of the erythroblasts before maturing to reticulocytes (dyserythropoiesis), and important erythrocyte morphology abnormalities. Clinically, CDA is characterized by anaemia, usually macrocytic, and iron overload. To date, five clinical forms of CDA have been described: CDA I, CDA II, CDA III, CDA IV and CDA with thrombocytopenia [17]. The first two (CDA I and CDA II) have an autosomal recessive hereditary pattern and the other two (CDA III and CDA IV) are autosomal dominant. Thrombocytopenia with CDA has an inheritance linked to the X chromosome. The two best known CDAs are CDA I (OMIM 224120) characterized by a marked dyserythropoiesis and frequent inter nuclear chromatin bridges and CDA II (OMIM 224100), characterized by marked dyserythropoiesis associated with erythroblastic binuclearity or multinuclearity, and the presence of erythroblasts with double cell membrane (**Figure 2**). CDA III (OMIM 105600) is a ultra-rare disease where the marked dyserythropoiesis is associated with severe nuclear size and chromatin morphological abnormalities that resemble an erythroleukemia or Di-Guglielmo disease.

CDA I is due to mutations of the CDAN1 gene or less frequently to mutations of the C15ORF41 gene, In a small percentage of cases the mutation is unknown.

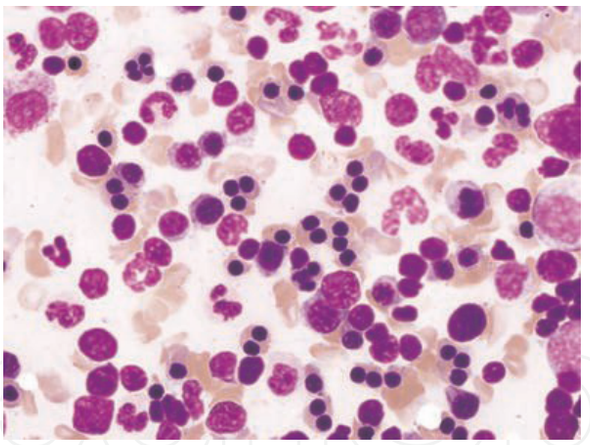

Figure 2.
Typical bone marrow picture of Congenital Dyserythropoietic Anaemia type II also known by hereditary erythroblastic multinuclearity with positive acidified serum lysis (HEMPAS) test.

In CDA II, the disease is due to mutations of the SEC23B gene and in CDA III, its most frequent gene mutation is the KIF23 gene.

5. Rare Anaemias due to red blood cell defects

RBC defects can be classified into two main groups: (a) Hereditary or intrinsic defects due to structural or functional abnormalities of RBC components haemoglobin (haemoglobinopathies), membrane (membranopathies) and enzymes of metabolism (enzymopathies), and (b) Acquired or extrinsic defects due to blood plasma or vascular abnormalities. In both cases the consequence is a haemolytic syndrome characterized by anaemia of variable severity associated with a compensatory increase of bone marrow erythropoiesis and of circulating reticulocytes. In addition to anaemia, the haemolytic syndrome is characterized by three main clinical manifestations 1. reticulocytosis, 2. splenomegaly and 3. jaundice [18].

5.1 Haemoglobinopathies

Haemoglobinopathies are the most frequent RBC defects in comparison with membranopathies and erythroenzymopathies that in many cases can be considered ultra-rare anaemias (prevalence <1 case per 10^6 inhabitants). All these diseases are more frequent in Southern Europe than in Central or Northern Europe, and their clinical expression is always an hereditary haemolytic anaemia [19, 20]. Haemoglobinopathies are the consequence of globin gene mutations that can alter the synthesis (thalassaemias) or the structure of haemoglobin (structural haemoglobinopathies). Its worldwide prevalence is around 269 million carriers, and in Europe there are risk populations, especially for thalassemia, which are located in the geographical regions surrounding the Mediterranean basin (Mediterranean Anaemia).

The most frequent haemoglobinopathy is HbS (OMIM 603903), prevalent in African populations due to the protection that it has offered against malaria. HbS is the result of substitution of valine for glutamic acid in the sixth position of the globin beta chain and in its homozygous form or combined with other haemoglobinopathies, is responsible for sickle cell disease (SCD) that consists in haemolytic anaemia associated with severe vaso-occlusive crises and pain due to multiple micro-infarcts [21]. These crises are triggered by hypoxia that decreases HbS

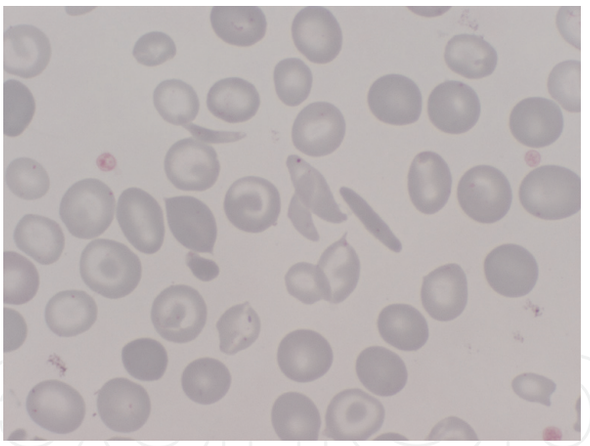

Figure 3.
Sickle-cells in a SCD patient with vaso-occlusive crisis.

solubility leading to a characteristic RBC shape distortion (sickle cell) and to a drastic decrease of deformability (**Figure 3**) Over the last 30 years, it has been observed a significant increase due to the immigration impact of populations from other geographical areas, mainly from Sub-Saharan African regions but also from Asia and Central America, where this disease is very prevalent. Currently, the neonatal screening programs allow an early diagnosis of the disease and its preventive treatment from the first years of life and, as a consequence, the frequency of complications have significantly reduced, and mortality during early childhood dramatically decreased [22]. There is no specific treatment for SCD, although in severe cases, the administration of hydroxyurea (HU) is recommended since, as the concentration of HbF increases, the frequency of vaso-occlusive crises, the need for transfusions, and especially the onset of the acute thoracic syndrome decrease. In addition to HbS, there are other structural haemoglobinopathies of clinical interest such as HbS, HbJ HbD and unstable haemoglobins that precipitate, and inclusion bodies or Heinz bodies are formed (**Figure 4**). Clinically, they present with a chronic haemolytic syndrome of variable severity and unlike the SCD, patients presents an autosomal dominant pattern of inheritance.

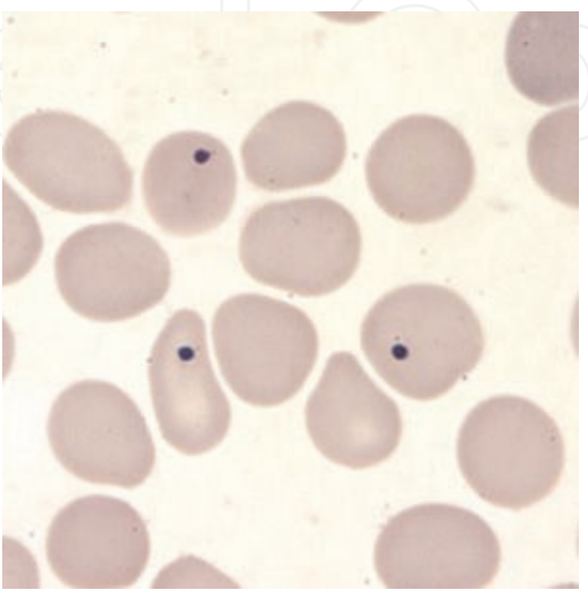

Figure 4.
Howell Jolly bodies in a patient with unstable haemoglobin.

5.2 Thalassaemias

Thalasaemias are due to the decrease in the synthesis of a globin chain (alpha or beta), due to absence, diminution or defective translation of specific messenger RNA (mRNA) caused by deletions or point mutations of the globin genes. While point mutations predominate in beta genes, large deletions are more frequent in alpha genes. According to the type of mutation and the intensity of the synthesis decrease, the severity of the clinical picture can be more or less intense [23]. In beta thalassemia the milder forms consist of a slight or moderate hypochromic and microcytic anaemia (thalassemia trait) whereas the more severe clinical forms can be classified as "thalassemia major" or "thalassemia intermedia" depending on the severity of the anaemia and the periodic transfusion requirements, respectively. In alpha thalassemia, as the genetic cluster has two genes, the mutation of a single allele, relatively common in Southern Europe in characterized by a moderate microcytosis (MCV around 80 fl) without anaemia alpha thalassemia trait, whereas if more than one allele is affected more severe forms of alpha-thalassemia occur The mutation of three alleles gives rise to the so-called haemoglobinopathy H, a clinical form very similar to intermediate beta thalassemia but with the presence of HbH or beta-globin tetramers, a result of the excess or imbalance of beta chains due to the decrease of alpha chains. The complete loss of the four alleles (homozygous alpha-thalassemia) is incompatible with life (hydrops faetalis).

Diagnosis of both forms of thalassemia is based on the data provided by the CBC and the study of haemoglobins by electrophoresis or high-performance liquid chromatography (HPLC). In the case of beta thalassemia trait an increase in the HbA2 fraction is always observed, whereas in alpha thalassaemia trait the haemoglobin pattern is normal and a molecular study is mandatory. An accurate family study is also very important to prevent diagnostic errors and the application of unnecessary treatments. In addition, an appropriate identification of the carrier condition allows the identification of couples at risk.

The treatment of severe clinical forms of β-thalassemias has been historically based on blood transfusions and iron chelation therapy. The only curative therapy currently available is allogeneic haematopoietic stem cell transplant (HSCT) from suitable donors. However, with the limited pool of suitable donors, HSCT remains unavailable for many thalassemic patients. They may instead benefit from globin gene therapy and other modalities, which exploit recent advances in understanding of globin gene regulation [24].

5.3 Membranopathies

Membranopathies are due to structural or functional defects of the RBC membrane proteins. In general, they are inherited as autosomal dominant pattern but transmitted with a recessive character. Hereditary spherocytosis (HS; OMIM 182870, 182,900, 270,970, 612,653, 612,690) is the most frequent cause of HHA in Caucasians due to a defect of membrane skeletal proteins that cause vesiculation and partial loss of the same with the decrease in surface/volume ratio and formation of spherocytes (**Figure 5**). Proteins affected in HS are beta-Spectrin (SPTB-1) Ankyrin and Band 3, and haemolysis occurs almost exclusively in the spleen, leading to frequent severe splenomegaly. Along with splenomegaly, several complications of HHA can be frequently observed in HS such as, intermittent jaundice and increased bilirubin pigments leading to premature gallstone formation, transient erythroblastopenia crisis due to parvovirus B19 infection, folic acid deficiency and torpid malleolar ulcers [25]. In general, physicians become more familiar with diagnosing HS in the newborn period, fewer neonates with HS will develop

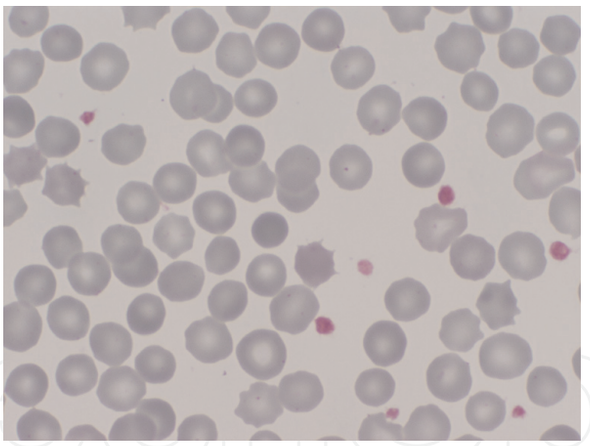

Figure 5.
Circulating peripheral blood spherocytes in a patient with hereditary spherocytosis.

hazardous hyperbilirubinemia or present to emergency departments with unanticipated symptomatic anaemia. The early suspicion, prompt diagnosis and treatment, using anticipatory guidance, will prevent adverse outcomes in neonates with HS [26].

The diagnosis of HS is based on the triad: (1) anaemia with jaundice, (2) severe splenomegaly and (3) spherocytosis, easily demonstrated by the peripheral blood morphological examination (**Figure 5**). Currently the diagnosis of HS can be easily performed by measuring RBC deformability and osmotic gradient ektacytometry (OGE) using the new generation LoRRca Osmoscan from Mechatronics [27]. Curves obtained with this device allow a clear distinction between hereditary spherocytosis and the other RBC hereditary membranopathies, elliptocytosis and xerocytosis (**Figure 6**). The implementation of the automated haematological analysers that determine the CCMH by means of a direct system, allows us to use this magnitude as a criterion of HS when it is increased in the presence of an elevated reticulocyte count. Finally, the use of the EMA-binding test technique is currently being implemented as a reference technique, especially in the diagnosis of HS, together with ektacytometry. This test is based on the measurement of the fluorescence intensity in RBCs after incubation with a fluorochrome, eosin-5-maleimide (EMA) that binds specifically to the anion transporter (Band 3) and decreases when Band 3 decreases.

Another hereditary membranopathy is hereditary elliptocytosis (HE, OMIM 109270, 130,600, 179,650, 225,450, 611,804), with milder clinical expression when compared to HS, but with the presence of more than 30% of circulating elliptocytes in peripheral blood (**Figure 7**). Like HS, HE is due to a skeletal protein defect, mainly alpha-Spectrin (SPTA-1) and Band 4.1 that alters the elasticity of the membrane preventing its recovery after elongation [28]. There is no loss of membrane and, therefore, CCMH is normal. OGE profile (Osmoscan) shows here a characteristic curve that is different from HS (**Figure 6**). In severe clinical forms of HE known as hereditary pyropoikilocytosis (HPP) the SPTA-1 gene mutation in heterozygous state is associated "in trans" with a SPTA-1 "Lely" mutation leading to severe HHA with markedly abnormal RBC morphology and decreased heat stability (**Figure 8**).

Finally, there is a very rare form of membrane disease called hereditary stomatocytosis (OMIM 194380, 185,000) which most relevant characteristic is the presence of RBC with an elongated central pallor instead of a round one (**Figure 9**). Its genetic and molecular mechanism is poorly understood, although it is known that in all forms there is a disorder of the permeability to sodium or potassium ions by which the RBC can be hydrated or dehydrated. These disorders of erythrocyte

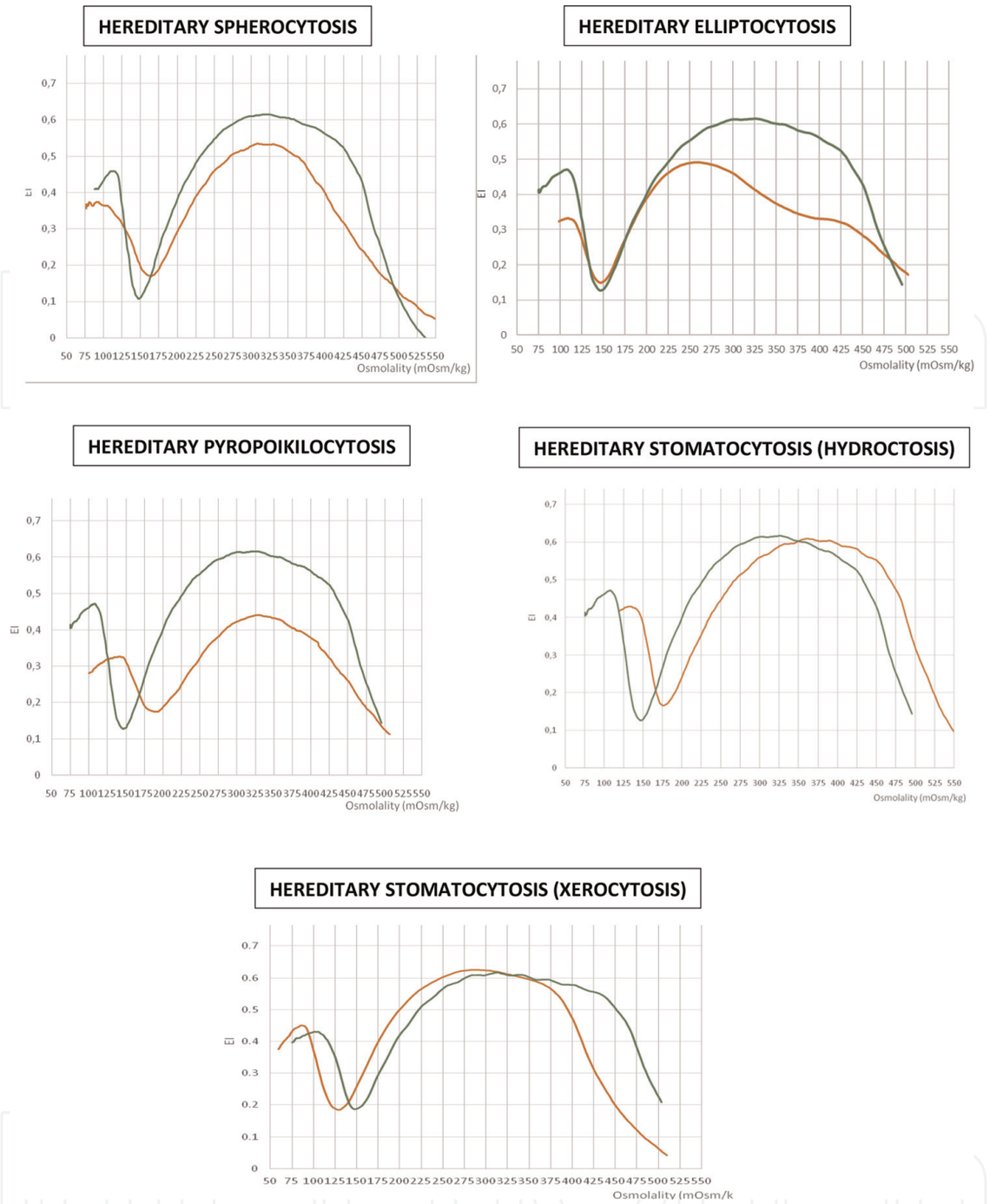

Figure 6.
Osmotic gradient ektacytometry (OGE) profiles from Osmoscan (LoRRca) in different hereditary membranopathies. EI: Elongation Index.

hydration are classified as primary, due to inherent disorders of erythrocyte volume regulation, and secondary, due to other disorders affecting the erythrocyte that secondarily influence cell hydration. In general, the degree of perturbation of water and ion content parallels the degree of haemolytic anaemia [29].

Overhydrated stomatocytosis (OHSt) or hereditary hydrocytosis refers to a rare, heterogeneous group of disorders with haemolytic anaemia and large numbers of stomatocytes on peripheral blood smear (**Figure 9**). OHSt is associated with markedly increased sodium permeability of the membrane from 10 to 40 times normal. This leads to a significant increase in intracellular sodium with a lesser decrease in intracellular potassium, resulting in increased total mono-valent cation content and increased intracellular water. Erythrocyte membranes from many OHSt patients

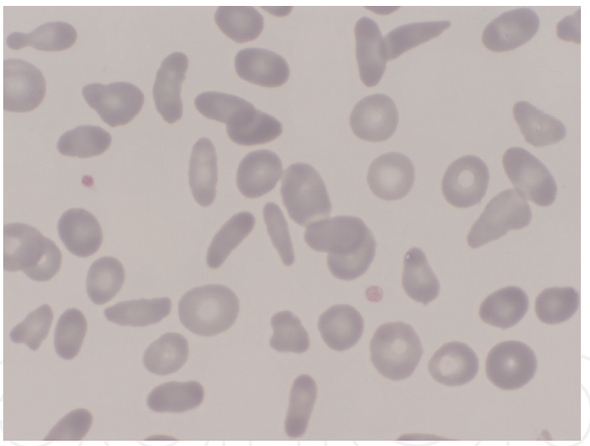

Figure 7.
Ellyptocytes in a patient with hereditary elliptocytosis.

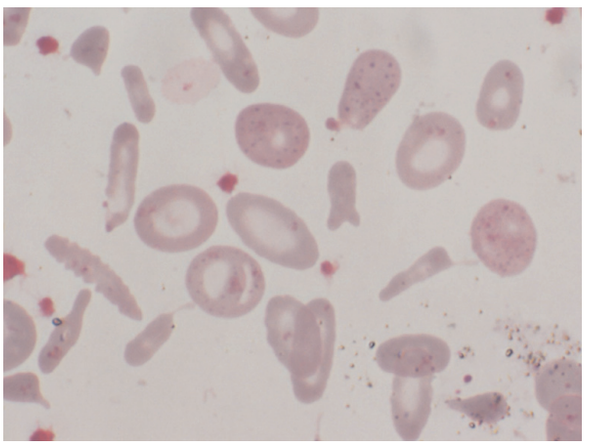

Figure 8.
Peripheral blood MGG stained examination of a newborn with hereditary pyropoikylocytosis (HPP).

lack stomatin, but mutations have not been found in the gene of affected patients and erythrocytes from stomatin knockout mice are normal. There is a variant of OHSt variant known as Cryohydrocytosis in which patient erythrocytes exhibit minimal to mild changes in cation leak at physiologic temperatures, but a marked increase in monovalent cation permeability at low temperature, typically 5°C. Erythrocytes demonstrate a sphero-stomatocytic morphology on peripheral smear. Heterozygous missense mutations in band 3, the anion exchanger (SLC4A1), have been identified in some cryohydrocytosis patients [29]. Dehydrated stomatocytosis or hereditary xerocytosis (HX) syndromes, are the most common primary disorders of erythrocyte hydration and are the most clinically heterogeneous. HX erythrocytes are dehydrated due to a cation leak, primarily of potassium, that leads to decreased potassium concentration. Because the cation leak is not accompanied by a proportional net gain of sodium and water, cellular dehydration ensues. Peripheral blood smear reveals few target cells, and occasional dessicytes (erythrocytes with haemoglobin puddled to one side), and rare stomatocytes. MCHC is increased (34–38 g/dL), and RBC osmotic fragility is decreased, both reflecting cellular dehydration [30]. Osmotic gradient ektacytometry (LoRRca Osmoscan) reflects a characteristic pattern of mixed reduced deformability index and dehydration given by a leftward shift of the minimal osmolality point (**Figure 6**).

When studied at the genetic level, in most cases of HX, mutations in PIEZO1 have been identified. PIEZO proteins are ion channels mediating mechanic sensory transduction in mammalian cells, and PIEZO1 is a large integral membrane protein

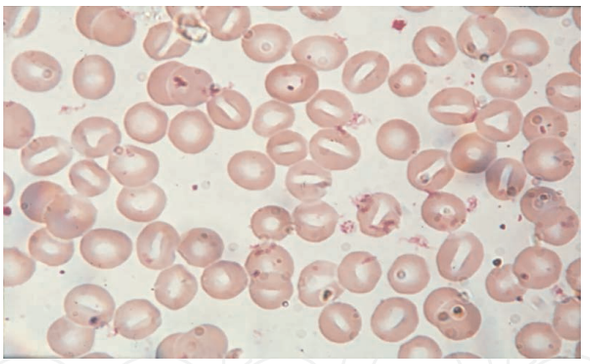

Figure 9.
Typical RBC morphology in a patient with hereditary stomatocytosis.

with numerous transmembrane domains that assemble into a homo-multimeric complex fully active as a mechanic sensitive cation channel [29]. A few HX patients do not have mutations in PIEZO1, but instead have mutations in the Gardos channel, encoded by KCNN4. The role of Gardos channel in normal erythrocytes has not yet been defined, butin HX-associated mutant KCNN4 channels demonstrate alterations in channel kinetics and trafficking. Clinically, HX patients with KCNN4 mutations experience variable degree of anaemia and RBC dehydration with typically more severe forms than in PIEZO1-mutant HX patients [29].

Treatment of HHA due to RBC membrane defects are always palliative, depending on the severity of anaemia. In HS, splenectomy allows a complete recover of haemoglobin concentration, whereas in the HE this recovery is only partial. In HS, splenectomy is not recommended because it facilitates thrombotic events due to an increased thrombophilia.

5.4 Erythroenzymopathies

Erythroenzymopathies are hereditary diseases due to a defect of RBC metabolism, in general an enzyme deficiency, that can be associated with an haemolytic crisis, a chronic haemolytic anaemia (HHA), neonatal cyanosis with metahaemoglobinemia or c) hereditary erythrocytosis. An association between enzymatic defect and HHA has been described in 14 of the 38 enzymes that make up the erythrocyte metabolism (**Table 4**). Some enzymatic defects produce haemolysis only under cellular stress produced by infections, oxidizing drugs or by the intake of fava beans. Other enzymatic deficiencies are associated with chronic non-spherocytic haemolytic anaemia (CNSHA). On some occasions, the expression of the deficiency is not restricted to the erythrocyte, but extends to other tissues, mainly the neurological, hepatic or muscular tissues, and a neuropathy, hepatopathy or myopathy, respectively, associated with anaemia appears. The RBC is especially sensible to enzymopathies because, unlike other cells, it cannot resynthesize the deficient enzymes because it lacks a nucleus and ribosomes. Enzymatic deficiencies occur when one of the enzymes of the reduced RBC metabolism is unstable and disappears very quickly, or has lost catalytic functionality and lacks activity. In the mature RBC there are two fundamental metabolic pathways: (1) the anaerobic glycolysis, by which glucose is used to generate ATP and (2) the aerobic pentose phosphate pathway (PPP), by which it eliminates any oxidative aggression to generate NADH and NADPH (**Figure 10**). ATP is used to meet energy requirements and NADH to reduce methaemoglobin. The NADPH is used to reduce the oxidized glutathione (GSH) which, in turn, is required to maintain the sulfhydryl groups of proteins in a reduced state and to detoxify hydrogen peroxide

Enzyme deficiency	Prevalence	Inheritance	Haemolysis	Comments
Hexokinase (HK)	Ultra-rare	Recessive	Yes (+++)	Low 2,3DPG level, poor tolerance of anaemia, 20 cases described.
Glucose phosphate isomerase (GPI)	Very rare	Recessive	Yes (++)	45 cases, 24 mutations re- ported; deficiency extended to leucocytes and platelets
Phosphofructokinase (PFK)	Very rare	Recessive	Variable	35 reported cases; variable degree of haemolysis and myopathy
Aldolase (ALD)	Ultra-rare	Recessive	Yes	3 cases reported
Triosephosphate isomerase (TPI)	Very rare	Recessive	Yes	Severe generalized disorder; widespread tissue involvement; neurologic, infectious and cardiac complications, 12 mutations described
Phosphoglycerate kinase (PGK)	Ultra-rare	X-linked	Yes, usually	Mental retardation and neurologic disturbances in hemizygous men; specific amino acid substitutions identified in 7 variants
Enolase (ENOL)	Ultra-rare	Dominant	Yes	3 cases described
Pyruvate kinase (PK)	The most common glycolytic defect	Recessive	Yes	First described and best studied defect, prototype for the group. 1:20,000 affected in the general population, 180 mutations described
Lactate dehydrogenase (LDH)	Ultra-rare	Recessive	No	7 cases described, myopathy with lack of M-subunit

Table 4.
Erythroenzymopathies of the glycolytic pathway.

(**Figure 11**). Enzymopathies have been described in both metabolic pathways. Glucose-6-phosphate dehydrogenase (G6PD, OMIM 300908, 134,700) is the most frequent antioxidant system, and shows hereditary transmission linked to sex. It is especially frequent in Africa, Asia and in the Mediterranean region (Greece and Italy, mainly) and, due to its polymorphic nature, it has many variants, among which the G6PD A-, predominant in black people, and the G6PD (−) Mediterranean, predominant in Caucasians. In G6PD A-forms, the enzyme is unstable but enzymatic activity is almost normal in reticulocytes, whereas in Mediterranean G6PD variants, the enzyme is even more unstable, and its activity is very low even in reticulocytes. This explains that in G6PD A-carriers, the acute haemolytic episode is self-limited and the recovery of the anaemia is faster than in the carriers of Mediterranean G6PD variants. Clinically, the G6PD deficiency occurs with haemolytic crisis sometimes associated with severe anaemia, in general triggered by the intake of oxidant substances, like fava beans (favism), or certain oxidizing drugs. Due to this, the carriers of a G6PD deficiency can remain asymptomatic for many years, until a contact occurs with the substances that may trigger the haemolytic crisis. Among the drugs that can induce haemolysis in G6PD deficiency, can be mentioned certain analgesics, sulphonamides, antimalarials, and antibiotics [31]. Favism, a severe haemolytic anaemia induced by fava beans ingestion or exposure to pollen from the plant, is the most frequent clinical manifestation in caucasians bearing the deficient G6PD Mediterranean variant, but also the G6PD A (−) variant (G66PD Betica). There are also ultra-rare forms of G6PD deficiency that do not obey to polymorphic variants but to

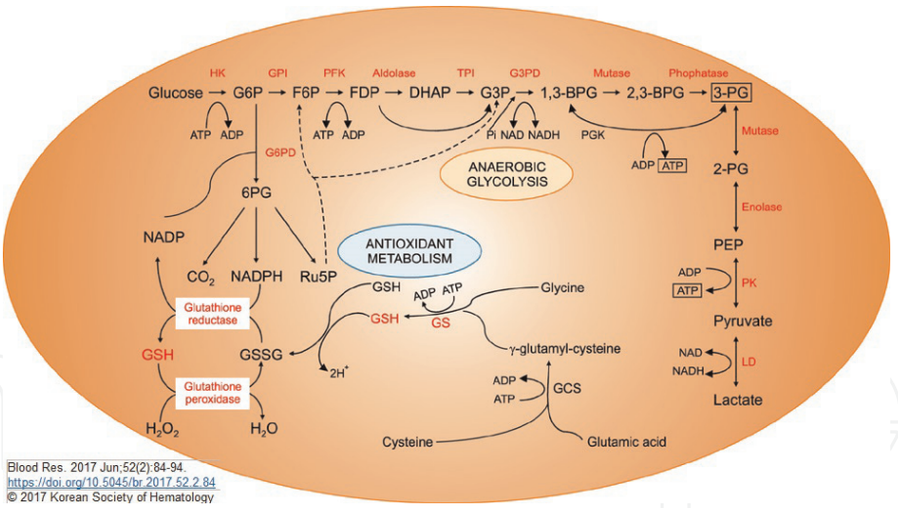

Figure 10.
Anaerobic glycolysis and antioxidant metabolic pathways of red blood cells. Abbreviations: BPG, bisphosphoglyceric acid; DHAP, dihydroxyacetone phosphate; F6P, fructose 6-phosphate; FDP, fructose-1,6-diphosphate; G3P, glycerol 3-phosphate; G3PD, glyceraldehyde 3-phosphate dehydrogenase; G6P, glucose 6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; GCS, glutamylcysteine synthetase; GPI, glucose-phosphate isomerase; GS, glutathione synthetase; GSH, glutathione; GSSG, glutathione disulfide; HK, hexokinase; LD, lactate dehydrogenase; NADP, nicotinamide adenine dinucleotide phosphate; PEP, phosphoenolpyruvic acid; PFK, phosphofructokinase; PG, phosphoglyceric acid; PGK, phosphoglycerate kinase; PK, pyruvate kinase; Ru5P, ribose-5-phosphate isomerase.

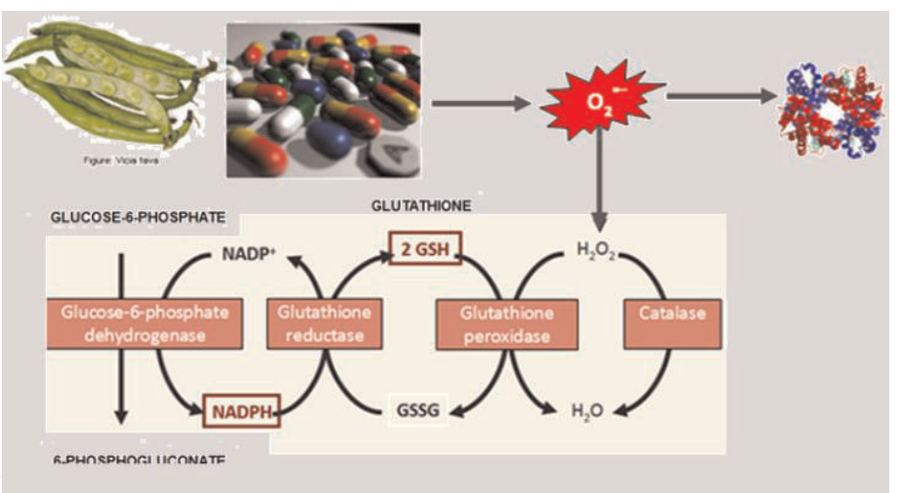

Figure 11.
Anti-oxidant system present in RBC to protect the cells against the oxidant threat generated by the ingestion of oxidant drugs of fava beans (favism).

sporadic variants that present with a chronic haemolytic syndrome (CSSHA). Other factors that can induce haemolysis in the G6PD deficiency are viral infections, especially influenza and hepatitis, diabetic ketoacidosis, and other different situations called of "stress." The diagnosis of G6PD deficiency is based on the clinical history, and the exclusion of the autoimmune mechanism through the negativity of the direct antiglobulin test (DAGT) or Coombs test. During the haemolytic crisis, the observation of the smear shows the presence of eccentrocytes or RBCs subjected to oxidative stress where haemoglobin is pushed off to one part of the cytoplasm (**Figure 12**).
For screening purposes, the fluorescent spot test is used based on demonstrating the formation of NADPH (fluorescent) from NADP (non-fluorescent) in the Beutler's G6PD fluorescence spot test or the reduction of methaemoglobin in the presence of methylene blue.

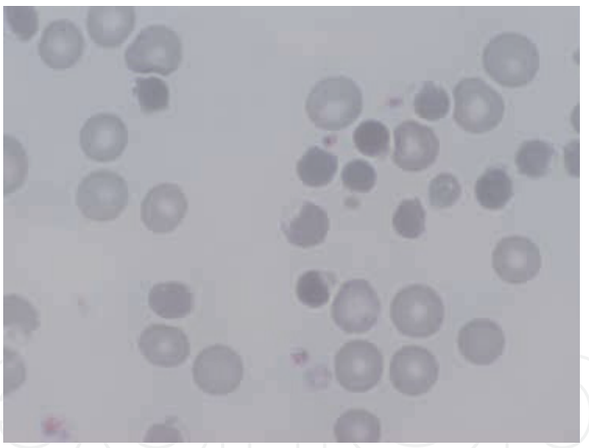

Figure 12.
Excentrocytes present in the blood of a patient with G6PD deficiency and haemolytic crisis.

The pyruvate kinase deficiency (PKD, OMIM 266200) is the most frequent enzyme of anaerobic glycolysis (Embden-Meyerhof pathway) and the most frequent cause of hereditary haemolytic anaemia (HHA), after hereditary spherocytosis [32]. It is an enzymopathy much less frequent than G6PD deficiency and its diagnosis requires the quantification of enzymatic activity in patient's haemolysates. Since haemolytic anaemia in PK deficiency is always due to mutations in the PKLR gene, leukocytes have a normal PK activity, so if they are not eliminated well when preparing the haemolysate, they can geopardise the result and mask the existence of a RBC PK deficiency (PKD). To avoid this important cause of error (false negatives), blood treated with anticoagulant must be filtered through a column of celulose-micro celulose in order to eliminate leukocytes and platelets. Also the presence of a high number of circulating reticulocytes, very frequent in newborns and children, can also give false negatives because the PK activity of the reticulocytes is much higher than that of the mature RBCs. Therefore, if in the presence of intense reticulocytosis a normal PK activity is obtained, the result should be confirmed by determining the quotient between PK activity and hexokinase (HK). HK is an enzyme whose activity also increases in the presence of reticulocytosis and, therefore, if this increase is much greater than that of PK, this enzyme deficiency is evidenced by a significant decrease in the PK / HK ratio [33].

Very recently a concise guide to PK deficiency for primary care providers, but also for haematologists, healthcare providers and medical students has been published [34]. There, the underlying PKD defect is explained in great detail together with its mode of inheritance, clinical manifestations, diagnostic procedures and an attempt for medical treatment.

6. Databases and epidemiology

Clinical epidemiology, originally confined to the global problems of infectious diseases, became, in the last 50 years, the fundament of today's evidence based medicine. No medical stakeholder is able to oversee the abundance of data describing prevalence, incidence, clinical patterns and health risks from all regions in the world. The majority of studies are concerned with common diseases, but there is still a paucity of data for rare diseases, which came in the scope of medical stakeholders in the last decade.

In RA, as in other rare and very rare disorders, only supranational networks can provide valid data. ENERCA has concentrated on data from the MS of the EU on a

subset of RA. However, it is often overlooked that social-economic conditions and medical practices are different in the different MS, resulting in different recognition of the epidemiological data studied [20]. Frequency is a general term to compare differences between the occurrence of a given disease, in the case of rare anaemias between populations defined by geographic regions or populations of different origin (such as the formerly called races) in a given region. The latter became of paramount importance with the ongoing immigration of people from the Mediterranean basin to North- and Middle Europe and from Asia and Africa to all MS, as mentioned above. The frequency is a useful parameter in any first approach of disease characteristics and a useful tool to decide on the methodology to estimate more precise issues. No one would challenge the fact, that thalassemia is much more frequent in the Mediterranean area or in immigrants within the Northern European countries. However, mathematically defined parameters are needed for research and health care planning. Prevalence, or more exactly prevalence proportion, is the main indicator used for any epidemiological studies in congenital diseases, representing the vast majority of conditions considered in disease category. Paroxysmal Nocturnal Haemoglobinuria (PNH) is an exception, with prevalence being of major significance, alike for the situation of cancers. For very rare anaemias included in ENERCA, usually period prevalence is measured, using a defined number of observation years rather than an index day as used for the so called point prevalence in common diseases. Another useful measure is the number of affected children of all live births in a given observation period; in this case, the period of definition of live birth (e. g. Days after birth) should be indicated, to avoid bias due to early mortality. An effective instrument to measure true prevalence a proportion is obtained with post-natal screening programs, as described for SCD. Ideally, all cases of a given disease or disease category in a population at time should represent the "true" prevalence. However, in the case of rare anaemias, the detection rate (number of detected/number of existing cases) is often less than 1. As shown by the work of ENERCA, there are strong indicators that the detection rates in the MS vary considerably depending on socio-economic conditions, and the same is true for the proportion of misclassifications. These data are of importance for any attempt to harmonize the diagnosis of rare anaemias in the European countries.

An inherent, unsolved problem of all registries is sustainability. Time trends, so important considering the influence of scientific as well as population changes due to both demographic evolution and migration can only be ascertained if sustainability can be guaranteed by resources of much longer duration than available today. Clinical trials, even though primarily directed to progress of therapeutic measures and often dependent on industrial interests are another source of epidemiological data. We hope that final EuorBloodNet standardization will provide solutions to many of these problems.

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