Genetic basis of Congenital Erythrocytosis: mutation update and online databases

Celeste Bento[1*#], Melanie Percy[2*#], Holger Cario[3*], Betty Gardie[4*], Tabita Magalhães Maia[1], Richard van Wijk[5], Silverio Perrotta[6*], Fulvio Della Ragione[7*], Helena Almeida[1], Cedric Rossi[8], François Girodon[8*], Maria Åström[9*], Drorit Neuman[10*], Susanne Schnittger[11*], Britta Landin[12], Milen Minkov[13], Maria Luigia Randi[14], Susana Rives[15], Leticia Ribeiro[1], Sylvie Hermouet[16*], Mary Frances McMullin[17*], on behalf of ECE-Consortium[§]

[1] Department of Hematology, Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal

[2] Department of Haematology, Belfast City Hospital, Belfast, Northern Ireland, UK

[3] Department of Pediatrics and Adolescent Medicine, University Medical Center Ulm, Ulm, Germany

[4] Inserm, UMR892, Université de Nantes, Nantes, France

[5] Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht, The Netherlands

[6] Dipartimento della Donna, del Bambino e di Chirurgia Generale e Specialistica, Second University of Naples, Naples, Italy

[7] Department of Biochemistry, Biophysics and General Pathology, Second University of Naples, Naples, Italy

[8] Laboratoire d'Hématologie, Centre Hospitalier Universitaire Dijon, France

[9] Departments of Medicine and Laboratory Medicine, Örebro University Hospital, Örebro, Sweden

[10] Department of Cell and Developmental Biology, Sackler Faculty of Medicine, Tel-Aviv University, Ramat-Aviv, Israel

[11] Munich Leukemia Laboratory, MLL, Munich, Germany

[12] Department of Clinical Chemistry, Karolinska University Hospital, Stockholm, Sweden

[13] Department of Hematology/Oncology, St. Anna Children's Hospital, Medical University of Vienna, Vienna, Austria

[14] Department of Medicine DIMED, University of Padua, Padua, Italy

[15] Department of Pediatric Hematology, Hospital Sant Joan de Déu de Barcelona, University of Barcelona, Spain

[16] Inserm, UMR892, Université de Nantes, Nantes, France

[17] Department of Haematology, CCRCB, Queen`s University, Belfast, Northern Ireland, UK

[*] Members of the MPN&MPNr-EuroNet (COST Action BM0902)

[§] Members of the consortium are listed in the acknowledgments

[#] These authors contributed equally to this work

Abstract

Congenital Erythrocytosis (CE), also called congenital polycythemia, represents a rare and heterogeneous clinical entity. It is caused by deregulated red blood cell production where erythrocyte overproduction results in elevated hemoglobin and hematocrit levels.

Primary congenital familial erythrocytosis is associated with low erythropoietin (Epo) levels and generally results from mutations in the erythropoietin-receptor gene (*EPOR*). Secondary congenital erythrocytosis arises from conditions which cause tissue hypoxia thus resulting in increased Epo production. These include hemoglobin variants with increased affinity for oxygen (genes *HBB*, *HBA1* and *HBA2*), decreased production of 2,3-biphosphoglycerate due to mutations in the *BPGM* gene, or mutations in the genes involved in the hypoxia sensing pathway (*VHL*, *EPAS1* and *EGLN1*). Depending on the affected gene CE can be inherited either in an autosomal dominant or recessive mode, with sporadic cases arising *de novo*.

Despite recent important discoveries in the molecular pathogenesis of CE, the molecular causes remain to be identified in about 70% of the patients.

With the objective of collecting all the published and unpublished cases of CE the COST action MPN&MPNr-Euronet developed a comprehensive internet-based database focusing on the registration of clinical history, hematological, biochemical and molecular data (http://www.erythrocytosis.org/). In addition, unreported mutations are also curated in the corresponding Leiden Open Variation Database (LOVD).

Key Words: Congenital Erythrocytosis, molecular pathogenesis, online databases

Background

Absolute erythrocytosis is defined by an increased red cell mass as reflected by hemoglobin and hematocrit values above the normal range. They can be either primary (intrinsic to the red cell) or secondary (extrinsic to the red cell) and can be acquired or arise from genetic alterations. Polycythemia Vera (PV) is the most common type of

acquired primary erythrocytosis with somatic mutations in the *JAK2* gene being responsible for almost 98% of the described cases (95% of the mutations involve the exon 14 with the p.Val617Phe, whereas only 3% involve the exon 12) (Cross, 2011). Acquired secondary erythrocytosis can develop from various diseases, such as cardiac, pulmonary or renal, or conditions of external hypoxia due to smoking and CO poisoning (reviewed by McMullin, 2008; Patnaik and Tefferi, 2009).

Primary erythrocytosis, also known as Primary familial congenital polycythemia (PFCP) is associated with a sub-normal serum erythropoietin (Epo) level. It is caused by a molecular defect in the hematopoietic progenitor cells. Previously diagnosed cases have been found to possess germline gain-of-function mutations in the Epo receptor gene (*EPOR*) (Huang et al., 2010 and Table 1).

In contrast, secondary congenital erythrocytosis (CE) is often characterized by inappropriately normal or raised serum Epo. It can be a consequence of tissue hypoxia, being caused by congenital defects such as hemoglobin variants with increased oxygen affinity due to mutations in the α- or β-globin genes (*HBB*, *HBA2*, *HBA1*) (Percy et al., 2009) or defective bisphosphoglycerate mutase (*BPGM*) leading to 2,3-biphosphoglycerate (2,3-BPG) deficiency (Hoyer et al., 2004). Secondary CE can also result from defects in components of the oxygen sensing pathway, and mutations in the genes that encode the hypoxia-inducible factor 2α (HIF-2α; gene *EPAS1*), HIF-prolyl hydroxylase 2 (PHD2, gene *EGLN1*) and the von Hippel-Lindau tumor suppressor (pVHL; gene *VHL*) have been reported (Lee and Percy, 2011) (Table 1).

Presently, over 160 mutations (more than 100 causing high affinity Hb variants and the others in the *EPOR* gene and genes of the oxygen sensing pathway) have been described

associated with CE. However in about 70% of the patients the molecular causes were not identified. Thus, this condition is referred to as idiopathic erythrocytosis (IE).

The oxygen-sensing pathway

Red blood cell production is regulated by the glycoprotein hormone Epo which is mainly synthesized in interstitial tubular kidney cells. Epo production is increased under conditions of hypoxia due to anemia or decreased cellular oxygen tension.

Under normal oxygen tension, the alpha subunits of the hypoxia inducible factor (HIF-1, 2 and 3) are hydroxylated by the dioxygenase PHD (PHD1, 2 and 3) and become a substrate for ubiquitination by pVHL, therefore being targeted for proteasomal degradation (Figure 1). Under low-oxygen conditions, PHD proteins are unable to modify HIF-α allowing it to escape pVHL recognition and subsequent degradation. HIF-α then forms an active transcriptional complex with HIF-β (ARNT) and up-regulates expression of more than 200 genes, with one of the target genes being Epo. The major HIF-α isoform involved in the regulation of *EPO* is HIF-2α, which also regulates genes involved in essential processes required for cell survival under low oxygen tension, such as heme synthesis (*ALAS2*), globin chains production (*GATA1*) and iron regulation (*TRF2*, *TF*) (Wenger et al., 2005, Zhang et al., 2011; Zhang et al., 2012; Lok and Ponka, 1999; Haase, 2010). Once Epo binds its cognate receptor there is initiation of an intra-cellular signaling cascade which inhibits apoptosis and simultaneously promotes the growth and differentiation of erythroid progenitors, thereby adjusting red blood cell mass to oxygen delivery requirements.

Erythrocytosis database

CE is a rare disease where clinical data on patients is sparse and information on clinical progression is not available. Moreover, although causative variants have already been identified in eight different genes, causal mutations remain to be identified in about 70% of the patients. A systematic repository may to aid clarifying the pathogenic manifestations of this disease, and, therefore, the CE working group (WG3), established within the framework of the COST (European Cooperation in Science and Technology) action BM0902 MPN/MPNr-Euronet, developed an internet based erythrocytosis database (www. erythrocytosis.org; Bento et al., 2006). In order to include all the clinicians and scientists enrolled in the diagnosis of CE an EU CE consortium, which will later be extended to other non-EU countries, was created.

The erythrocytosis database aims to collect and share clinical and laboratory information on patients with absolute erythrocytosis, either idiopathic or with an already established molecular diagnosis. The database, accessible only after validated registration, contains different types of data, including clinical, biochemical and molecular, which then can be annotated. To unauthenticated users only a landing page is available, explaining the aims of the registry and the guidelines of the EU CE consortium (ECE-C; Figure 2).

As such, www.erythrocytosis.org is a comprehensive and reliable genotype–phenotype database that will fulfill the needs of clinical practitioners, who require reliable markers for disease diagnosis and prognosis. This database will also assist researchers who aim to establish genotype-phenotype correlations for the new mutations or genes discovered. Clinical and laboratory data collected at different times can be registered and compared. Statistical correlations can be achieved by matching all the required parameters. This

database will provide a knowledge base on which to develop guidelines and update diagnostic algorithms for new genetic testing and will accelerate the understanding of the clinical and molecular mechanisms underlying erythrocytosis. It is not a repository of the CE mutations already registered in the literature. Complete information on previously reported mutations can be obtained from the comprehensive Leiden open variation database (LOVD, www.lovd.nl; Fokkema et al., 2011). Since the curators of the erythrocytosis database are also curators of the CE LOVD databases there is an option for new reported mutations to be also reported and inserted into the LOVD database as a personal communication.

Current status of the database

At the time of submission only patients with mutations already identified have been registered in the database. It is planned that in a second phase data idiopathic erythrocytosis patients will be added.

Of the 158 patients included in the database, 40 are carriers of a high affinity Hb variant, 26 are heterozygous for an *EPOR* mutation, 38 are homozygous or compound heterozygous for a *VHL* mutation, 15 are heterozygous for a mutation in *VHL*, 15 are heterozygous for an *EPAS1* mutation and 24 are carriers of an *EGLN1* mutation (Table 2). Of these, three *EPOR*, three *VHL* and four *EGLN1* mutations are reported here for the first time (Figures 3-6).

High Oxygen affinity hemoglobin variants

Genes *HBB*, *HBA2*, *HBA1*

The genes that encode the alpha (*HBA)* and beta (*HBB*) globin chains of hemoglobin, are located on chromosomes 16 (locus 16p13.3) and 11(locus 11p15.4) respectively. There are two *HBA* genes (*HBA2* and *HBA1*; MIM 141850 and 141800) that have arisen by gene duplication. Both genes encompass three exons and encode a 142 amino acid protein (MW~15 kDa) although the mRNA *HBA2* transcript is 605 base pairs (bp) while the mRNA *HBA1* transcript is 577 bp long.

The *HBB* gene (MIM 141900) is also comprised of three exons with a transcript of 754 bp long which encodes a 147 amino acid protein (MW~16 kDa).

The first described molecular defect associated with CE was in an 81-year-old man with a hemoglobin of 19.9 g/dl who was seen at the Hematology Clinic in Johns Hopkins Hospital by Samuel Charache (Charache et al., 1966). A thorough family study revealed 15 other members with increased hemoglobin levels, all of them showing an abnormal hemoglobin band on electrophoresis. In addition, the oxygen dissociation curve was significantly displaced to the left, indicating increased oxygen affinity of hemoglobin. Structural analysis established that there was an alpha-chain variant with a substitution of leucine for arginine at position 92 and this variant was subsequently called Hb Chesapeake.

Since then more than 100 mutations have been described in the globin genes, with the majority being present at the *HBB* locus, that give rise to high oxygen affinity hemoglobin variants. Mutations are dominantly inherited and there are only a few cases reported arising *de novo*.

Most of the high-affinity variants described thus far have substitutions at one of three regions that are crucial for hemoglobin function 1) the α1β2 interface; 2) the C-terminal end of the β-chain; 3) the 2,3-DPG binding site (reviewed in Thom et al., 2013)

All the described Hb variants are compiled in a complete and updated database, Hb Var (http://globin.bx.psu.edu/hbvar/menu.html). Since only patients with new mutations are registered, it is not possible to estimate the real incidence and prevalence of the high affinity hemoglobin variants.

BPGM

The *BPGM* gene (MIM 613896; locus 7q33) extends over 22 kb and contains 3 exons. The first exon (130 bp) is not included in the coding DNA sequence (CDS) region, whereas the other two (1753 bp) encode a protein of 259 amino acids (MW ~30 kDa).

The BPGM enzyme is important in the regulation of hemoglobin's affinity for oxygen because it controls the level of 2,3-BPG, which is generated in the Rapoport-Luebering Shunt, a bypass of glycolysis. When 2,3-BPG is bound to hemoglobin it decreases hemoglobin's affinity for oxygen (Benesch et al.,1969). Consequently, it allows the efficient off loading of oxygen at respiring tissue. Deficiency of BPGM enzyme results in reduced synthesis of 2,3-BPG and red cell production is increased to compensate for less available oxygen.

Reported cases in the literature of erythrocytosis due to BPGM mutations are very rare with only three variants being described. Compound heterozygosity for a missense mutation c.268C>T (p.Arg90Cys) and a small deletion c.61delC (p.Arg21Valfs*28) was found in four members of the same family (Rosa et al. 1978; Lemarchande et al., 1992).

Hoyer et al. (2004) reported a patient homozygous for a missense mutation c.185G>A (p.Arg62Gln).

EPOR

The *EPOR* gene (MIM 133171; locus 19p13.2) encodes the Epo receptor protein, which is a member of the cytokine receptor family. *EPOR* is composed of 8 coding exons.

The primary transcript is 2056 bp long and encodes a protein of 508 amino acids (MW~66 kDa). Alternatively spliced forms of the Epo receptor have been identified, one of which has a truncated cytoplasmic domain. The shorted transcript is expressed at high levels in immature erythroid progenitor cells. In contrast, the expression of the full-length receptor increases as progenitor cells mature (Nakamura et al., 1992).

The first mutation found in the *EPOR* gene was in a previously very successful Finnish sportsman and 29 family members as described by de la Chapelle et al. (1993). Since then more than 22 heterozygous variants have been found in patients with CE. All of these mutations are located in exon 8, which encodes the C-terminal negative regulatory domain of the protein. In total, 18 are *frameshift* mutations (due to small deletions or insertions) or *nonsense* mutations leading to cytoplasmic truncation of the receptor and loss of the C-terminal negative regulatory domain (Figure 3). These mutations induce a gain-of-function and are associated with Primary Congenital Familial Erythrocytosis (PFCP), which is also known as familial erythrocytosis type 1 (OMIM 133100; Table 1). Of the remaining variants, three are missense mutations (c.1462C>T, c.1460A>G, c.1140G>A) in which the association with erythrocytosis has not yet been established.

VHL

The *VHL* gene (MIM 608537) is located on chromosome 3 (locus 3p25.3) and spans 10 kb. The *VHL* gene encodes a 4.7 kb mRNA translated from two translational initiation sites (+1 and +54). The larger protein consists of 213 amino acids (pVHL30 MW~30kDa), whereas the shorter protein consists of 160 amino acids (pVHL18), both are functionally active (Iliopoulos et al., 1998). pVHL is the substrate recognition subunit of an E3 ubiquitin ligase and interacts with elongin C and B and Cullin 2, in a complex referred as VCB-CUL2.

There are more than 400 germline mutations in the *VHL* gene that have been first described associated with the VHL disease (OMIM 193300) (Nordstrom-O'Brien et al., 2010). VHL disease is an autosomal dominantly inherited syndrome predisposing to the development of a panel of benign and malignant, highly vascularized tumors including hemangioblastomas, pheochromocytomas (or paragangliomas) and renal cell cancer, but VHL disease is outside the ambit of this article. The association of VHL disease with erythrocytosis was recently reviewed by Capodimonti et al. (2012). The association of CE *VHL* mutations with tumors will be discussed below in the section entitled “Risk of tumor development”.

The first loss-of-function mutation in the *VHL* gene associated with CE was found in the Chuvash autonomous republic of Russia where polycythemia is an endemic disorder. Chuvash polycythemia was found to be caused by a homozygous c.598C>T (p.Arg200Trp) *VHL* mutation (Ang et al., 2002a, 2002b). Later, homozygosity for the *VHL c.598C>T* mutation was also observed in non-Chuvash patients and 16 additional *VHL* variants associated with CE have been described (Figure 4). Four of them presented in the homozygous state, whereas the other cases were compound

heterozygous or heterozygous. Although *VHL* associated erythrocytosis (CE type 2, OMIM 263400; Table 1) is considered a recessive disease some cases have been described where only one mutation was detected (see Carriers of *VHL* mutations with CE).

EGLN1 (PHD2)

There are three PHD isoenzymes (PHD1, PHD2 and PHD3), but PHD2 was found to be the key enzyme in catalyzing the prolyl hydroxylation of HIF-α, using oxygen as a co-substrate (Kunz and Ibrahim, 2003; Percy et al., 2006). PHD2 is encoded by the *EGLN1* gene (MIM 606425), which is located on chromosome 1q42.1, and it is comprised of five exons. The *EGLN1* mRNA is 7.097 bp long and translates into a 426 amino acid protein (MW~46 kDa).

Loss-of-function mutations in *EGLN1* cause CE type 3 (OMIM 609820) (Table 1) with autosomal-dominant inheritance. Mutations were first described by Percy et al. (2006) who identified a heterozygous c.950C>G transversion in two generations from one family (3 family members). The mutation resulted in a p.Pro317Arg substitution in a highly conserved region of the protein (Figure 5). *In vitro* functional expression studies showed that the mutant protein had significantly decreased enzyme activity. Epo levels in the son and daughter were inappropriately normal, suggesting deregulated Epo production. Since then more than 22 patients were found to carry mutations in this gene, all of them heterozygous for one of the 16 previously reported mutations: 12 missense, 2 nonsense, 1 small deletion and 1 small duplication (Figure 5). One of the missense mutations, c.471G>C (p.Gln157His) was found to co-exist with the *JAK2* p.Val617Phe

somatic mutation, the latter probably being the cause of the disorder. Meanwhile, the c.471G>C mutation has been categorized as a SNP (rs61750991) with a frequency of around 2% in the normal population although some studies refer to a higher frequency (Astuti et al., 2011; Ladroue et al., 2012). Interestingly, one particular mutation (p.His374Arg) has been described in a patient with an erythrocytosis associated with a recurrent paraganglioma (Ladroue et al., 2009).

***EPAS1* (HIF-2α)**

The HIF transcription factor has three isoforms, HIF-1α, HIF-2α and HIF-3α. HIF-1α was first identified as a mediator of Epo induction in response to hypoxia *in vitro* (Wang & Semenza, 1995), however HIF-2α was later confirmed as the primary transcription factor that induces Epo expression (Scortegagna et al., 2003; Warnecke et al., 2004; Hickey et al., 2007, Percy et al., 2008a). The degradation of HIF-2α occurs via the hydroxylation of the residues Pro 405 and Pro 531.

The *EPAS1* gene (MIM 603349), which encodes the transcription factor HIF-2α, is located on chromosome 2p21, contains 16 exons and spans at least 120 kb. The 5160 bp long *EPAS1* mRNA is translated into an 870 amino acid protein (MW~96 kDa).

Gain-of-function mutations in exon 12 of *EPAS1* are another cause of familial erythrocytosis (type 4, OMIM 611783), showing autosomal-dominant inheritance (Figure 6). The first *EPAS1* mutations found in erythrocytosis patients were the missense mutations p.Gly537Trp, p.Gly537Arg, p.Met535Val and p.Pro534Leu (Percy et al. 2008a, 2008b, Percy et al., 2009). Martini et al. (2008) described another pathogenic mutation, p.Met535Ile, and more recently, three additional missense mutations have been described, p.Asp539Glu, p.Met535Thr, p.Phe540Leu (van Wijk et

al., 2010, Percy et al. 2012). In total, 22 patients (8 sporadic cases and 4 families) have been reported harboring mutations in this gene, all of them heterozygous. Recently Lorenzo et al. (2012) identified a germline heterozygous missense mutation c.1121T>A (p.Phe374Tyr) in exon 9 in a polycythemic patient who developed pheochromocytoma/paraganglioma. This variant was already reported in the NCBI dbSNP database (rs150797491) with a minor allele frequency of 0.1%. Somatic mutations associated with paraganglioma and erythrocytosis have been described (Zhuang et al., 2012;Yang et al., 2013) but they are not within the scope of this paper.

Biological Significance

The identification of CE causal mutations in the HIF pathway genes has established the PHD2:HIF-2α:VHL pathway as the key regulator of adaptation and survival of both cells and the whole organism to hypoxia through Epo regulation (Lee and Percy, 2011).

It was previously noted that in some instances VHL disease was accompanied by erythrocytosis but these secondary erythrocytoses were due to Epo production by the tumor itself and disappeared after tumor removal. Hence, they resolved after tumor removal.

The first insight into CE came from the studies on Chuvash polycythemia patients where the p.Arg200Trp mutation in the *VHL* gene led to an autosomal recessive form of erythrocytosis. The *VHL* p.Arg200Trp loss-of-function mutation results in diminished ubiquitination of the HIF transcription complexes and less proteasomal regulation in normoxia.

Further studies screening individuals with erythrocytosis for defects in HIF-1α, HIF-2α and the three isofroms of PHD hydroxylases detected mutations in only PHD2 and HIF-2α genes (Percy et al., 2006; Percy et al., 2008a; Percy et al., 2008b). These results indicated that PHD1 and PHD3 isoforms were unable to compensate for the loss of PHD2 function and there was no redundancy in the oxygen sensing pathway. Furthermore, the different isoforms of HIF and PHD exhibited different specific functions.

The HIF-1 transcription complex was described as the main regulator of Epo from binding studies and for a decade this was believed to be the case. However, the results from erythrocytosis studies caused a paradigm shift resulting in HIF-2α now being recognized as the main isoform that controls Epo. This was borne out by mice and RNA interference studies (Scortegagna et al., 2003; Warnecke et al., 2004). It is now acknowledged that HIF-1α and HIF-2α regulate different target genes (reviewed by Mole and Ratcliffe, 2008).

Inherited mutations in HIF-2α are all located close to the site of prolyl hydroxylation at Pro531 and this region is crucial for the binding of PHD2 for hydroxylation and VHL for ubiquitination of HIF-2α (Furlow et al., 2009). Functional analysis of a series of HIF-2α mutations has shown that in most cases the binding of both PHD2 and VHL is decreased, except for the p.Met535Val mutation, whereas VHL binding is retained. Thus diminishing PHD2 binding alone is sufficient to cause impairment of the oxygen sensing pathway and dysregulation of Epo synthesis.

At the physiological level, VHL mutations have more profound consequences, not just affecting hemopoiesis but also metabolism and exercise capacity, as both HIF-1α and HIF-2α proteins are stabilized in normoxia (Formenti et al., 2010). Consequently, a broader range of target genes is up-regulated in subjects carrying VHL mutations compared to those with HIF-2α mutations, further highlighting the differing functions of the HIF-α isoforms.

Clinical Significance of Mutation Identification

The elevated number of red blood cells and high hematocrit with a consequent hyperviscosity, may result in CE patients presenting with symptoms and signs ranging from headaches, dizziness, epistaxis and exertional dyspnea to pruritus after bathing. Moreover, thrombotic and hemorrhagic events leading to premature morbidity and mortality have been reported. Clinical symptoms are effectively relieved by phlebotomy, but the increased risk of cardiovascular morbidity is not necessarily ameliorated by maintaining a normal hematocrit (Van Maerken, 2004).

The Chuvash cohort, who is homozygous for the *VHL* R200W mutation, has been most extensively studied clinically. Homozygous patients have been compared with a spouse control group and age sex matched community controls some of whom were *VHL* heterozygotes. Survival in the Chuvash patients was found to be reduced compared to the control groups and there were higher rates of arterial and venous thromboses (Gordeuk et al., 2006). No increase in cancers was seen in the Chuvash polycythemia cohort. Clinically those with Chuvash polycythemia had lower blood pressures than the heterozygotes while the heterozygotes had lower blood pressures than the controls

(Gordeuk et al., 2004). Homozygotes were observed to have more venous varicosity than control groups. There have been no reports of CE type 2 (*VHL* mutations) patients developing tumors, except for two cases of isolated hemangioblastoma (Woodward et al., 2007).

Cardiopulmonary physiology has been studied in Chuvash polycythemia patients compared to two control groups. Participants were studied at baseline and then subjected to hypoxia. Mild hypoxia induced a greater increase in ventilation in the Chuvash patients compared to the controls and they did not tolerate moderate hypoxia. They had abnormally high pulmonary artery pressures and hypoxia provoked a further abnormal rise. Physiological studies showed that Chuvash patients appeared to be in a situation characteristic of acclimatisation to the hypoxia resulting from high altitude (Bushuev et al., 2006; Smith et al., 2006). These patients should be regularly monitored for cardiopulmonary function.

Studies in the original Chuvash population revealed that the occurrence and severity of thromboembolic events was independent of the intensity of a possible phlebotomy treatment (Gordeuk et al., 2004). Therefore, particularly in patients with pulmonary hypertension (PHT) discernable risks of phlebotomy treatment have to be calculated very carefully with regard to a possible negative influence of iron deficiency on PHT (Craig et al., 2012; Sable et al., 2012).

EGLN1 and *EPAS1* mutations are mostly described in single case reports and there is little clinical information available. However, there are a few thromboembolic events reported occurring at young ages which are likely to be of significance in association

with the mutations (Percy et al., 2008a). Pulmonary hypertension has also been described in individuals with the *EPAS1* p.Gly537Arg mutation (Gale et al., 2008). The underlying physiological changes are similar to those observed in patients with Chuvash polycythemia (Formenti et al., 2011)

Several cases with *EGLN1* and *EPAS1* mutations developed paraganglioma and this will be discussed in the section entitled "Risk of tumor development in patients with CE".

CE patients with high oxygen affinity hemoglobins usually are generally asymptomatic but hyperviscosity symptoms and thromboembolic episodes have been reported and related to the high hematocrit (Fairbanks et al., 1971; Weatherall et al, 1977)**.** However, phlebotomy is not simple solution since erythrocytosis in these patients is primarily a requirement due to general tissue hypoxia. Therefore, phlebotomy treatment will be limited to single symptomatic events. In severe symptomatic cases regular exchange transfusion may have to be considered. Cases have been described showing unusual incidences of spontaneous abortion in female carriers caused by the lower oxygenation of the fetus resulting from either the alteration in the physiological oxygen gradient affinity between foetal and maternal blood or by placental infarction caused by the high viscosity of the mother's blood (Koller et al., 1980, Bento et al., 2000).

In conclusion, the raised hematocrit and increased viscosity associated with CE may lead to a number of clinical complications including increased thromboembolic events at young ages but in the absence of good clinical data and follow up it is difficult to obtain a true picture of the clinical situation. At present, it is not possible to make clear treatment recommendations. However, the identification of the underlying genetic

defect aids avoidance of possible pitfalls in the treatment (e.g. phlebotomy in hemoglobinopathies), organization of adequate monitoring (e.g. for PHT in *VHL* and *EPAS1* cases) and to counsel the patient.

Carriers of *VHL* mutations with CE

Although CE type 2 is considered a recessive disease the occurrence of individuals heterozygous for *VHL* mutations with erythrocytosis has been described. Eleven independent cases of erythrocytosis patients heterozygous for *VHL* mutations were reported in the literature. In contrast, other carriers of the same VHL mutation exhibited normal hematological parameters. In the case of heterozygous carriers with erythrocytosis the presence of other *VHL* mutations or a *VHL* null allele or deletion that could affect the apparently wild-type *VHL* allele had been ruled out and no mutations in the other genes associated with CE were found (Pastore et al., 2003; Bento et al., 2005; Percy et al., 2003a; Randi et al., 2005; Cario et al., 2005; Perrotta et al., 2006; Percy et al., 2007).

In addition 15 patients with erythrocytosis but only heterozygous *VHL* mutation are registered in the erythrocytosis database (Table 2). It is interesting that in two of these cases, twin brothers heterozygous for the p.Arg200Trp mutation, the wild type allele showed an unexplained low expression of *VHL* (data not shown).

The use of next generation sequencing (NGS) will be a useful tool in the identification of novel candidate genes associated with the development of erythrocytosis.

Risk of tumor development in patients with CE

The risk of patients with germline mutations in the HIF pathway (*VHL*, *EGLN1*, *EPAS1*) to develop tumors need to be considered, knowing the crucial role that hypoxia plays during tumorigenesis.

In inherited cancer diseases associated with the loss of tumor suppressor genes, the mechanisms of tumor development imply that the first event leads to a loss of function sufficient to induce a selective pressure, which results in the loss of the second allele. Germline heterozygous mutations in the *VHL* gene predispose to the development of multiple tumors which have subsequently lost the remaining wild type allele.

Concerning the *VHL* p.Arg200Trp mutation, the heterozygous carriers never develop malignant tumors and parents of the patients with Chuvash polycythemia are healthy. Two rare cases have been described with erythrocytosis that developed hemangioblastomas, which are benign tumors (Woodward et al., 2007). It is possible to hypothesize that the p.Arg200Trp mutation is not sufficiently deleterious to allow a selective pressure and initiate tumorigenesis. Indeed, the VHL p.Arg200Trp mutation is considered as less severe than classical VHL mutants (Ang et al., 2002; Rathmell et al., 2004) and a recent comprehensive functional study of this mutant showed that it is similar to the wild type protein (Gardie et al., in preparation). Therefore, the risk of patients carrying the p.Arg200Trp mutation to develop malignant tumors can be estimated as very limited. However, stringent follow up is recommended for patient carring other *VHL* mutations in which the severity of the loss of function has not been precisely determined (Figure 4).

The *VHL* mutations associated with CE are all missense mutations, except for one truncating mutation, VHL p.Glu10X. This particular mutation is located between the

two translation initiation codons and has the capacity to produce a pVHL19 isoform still able to regulate HIF.

Regarding the other genes of the HIF pathway mutated in erythrocytosis (*EGLN1*, *EPAS1*), the evaluation of the risk of developing tumors is more complicated because of the restricted number of described cases and because of the closely related isoforms (PHD1, 3 and HIF-1α) which are theoretically able to compensate for the dysregulation of HIF. Nonetheless, the follow up of the patients carrying such mutations is highly recommended. Indeed, paragangliomas (tumors of the VHL disease spectrum) have already been described in patients carrying a particular *EGLN1* mutation (H374R) and a *EPAS1* mutation (p.Phe374Trp). The study of the patient with the *EGLN1* p.His374Arg mutation indicated there was severe loss of function compared to mutations associated with erythrocytosis (Ladroue et al., 2012). Furthermore, examination of the paraganglioma from the patient indicated a loss of the remaining *EGLN1* wild type allele in the tumor (Ladroue et al., 2008). *EGLN1* is therefore a potential tumor suppressor gene as already been suggested (Kato et al., 2006; Lee, 2008).

Regarding *EPAS1* mutations, it should be noted that none of the germline mutations identified in patients with CE target the main hydroxylated prolines (Pro405 and Pro531). Nonetheless, mutations targeting the Pro531 have been described, but only at the somatic level in four cases of pheochromocytomas/paragangliomas (Favier et al., 2012; Toledo et al., 2013). These observations suggest that total and excessive activation of HIF-2α may be necessary for tumorigenesis.

Performing accurate comparative functional studies of the HIF pathway mutants is required in order to evaluate the risk of the carriers to develop tumors.

Diagnostic Strategies

When diagnosing a patient with erythrocytosis it is important to exclude acquired secondary (pulmonary, renal and cardiac) or acquired primary (PV due to *JAK2* mutations) causes.

The family history and the determination of serum Epo levels are very useful in the decision regarding which molecular tests should be performed first. If available, determination of p50 (percentage at which Hb is half saturated with oxygen) can be helpful in establishing the presence of a hemoglobin variant with high oxygen affinity.

Sequencing of the candidate genes is mandatory for a definitive diagnosis. Based upon the serum Epo level and familial data, it is possible to establish an algorithm to decide which genes should be sequenced in each case of erythrocytosis (Figure 7). A comparable algorithm with a specific focus on diagnostics in affected children and adolescents has been published recently (Cario et al., 2013).

Future Prospects

Significant advances have been made during the past decade in the CE field with the identification of causal mutations in the *EPOR* gene and the elucidation of the genes directly implicated in the hypoxia sensing mechanism (*VHL*, *EGLN1* and *EPAS1*). Presently, over 160 mutations have been associated with CE but despite this about 70% of the CE patients, and 12-35% of PFCP cases, still remain unexplained at the molecular level. The absence of erythrocytosis in a child heterozygous for a deleterious nonsense *EPOR* mutation (Kralovics et al., 1998) and the observation of individuals heterozygous for *VHL* mutations with erythrocytosis confirm that other genes or epistatic factors must be implicated in the clinical manifestation of CE. The coming use

of next-generation sequencing is expected to further expand the number of genes involved in CE.

With the implementation of the internet-based erythrocytosis database, it is hoped that it will allow the establishment of clinical and genotype–phenotype correlations in larger groups of individuals.

Mutation Nomenclature and Accession Numbers

The mutation nomenclature used in this update follows the guidelines indicated by Human Genome Variation Society (HGVS) [den Dunnen and Antonarakis, 2003]. Mutation descriptions have been checked using the Mutalyzer program (https://mutalyzer.nl/). Nucleotide numbering is based on GenBank reference sequences NM_000518.4 for *HBB*, NM_000558.3 for *HBA1*, NM_000517.4 for *HBA2*, NM_199186.2 for *BPGM*, NM_000121.3 for *EPOR*, NM_000551.3 for *VHL*, NM_022051.2 for *ENGL1*, NM_001430.4 for *EPAS1*.

Web Resources

The URLs of resources used and/or cited in this work are the following:

Single Nucleotide Polymorphisms database: http://www.ncbi. nlm.nih.gov/SNP/;

Entrez Gene database: http://www.ncbi.nlm. nih.gov/gene/; Online Mendelian Inheritance in Man (OMIM): http://www.ncbi.nlm.nih.gov/omim; LOVD databases: http://www.lovd.nl/2.0/; HbVar (http://globin.bx.psu.edu/hbvar)

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