Congenital Erythrocytosis and Hereditary Thrombocytosis

Clinical presentation, diagnosis, treatment and follow-up
A practical guide with clinical cases

2015

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Presentation

The possibility that patients may present with erythrocytosis – the excessive production of red blood cells – or thrombocytosis – the excessive production of platelets – has been known for at least a century. Different types of erythrocytosis and thrombocytosis (primary, secondary) and their complications (thrombosis, myelofibrosis, malignant transformation and more rarely, bleeding) have been extensively described, and the most frequent causes of secondary erythrocytosis (insufficient supply of oxygen to tissues) and thrombocytosis (inflammation, deficiency in primary hemostasis) were identified. In the late 1950s and in the 1960s, it was recognized that certain patients were born with familial (hereditary) defects leading to erythrocytosis (Auerback ML *et al*., 1958; Cassileth PA *et al*., 1966) or thrombocytosis (Beretta Anguissola and Prato V, 1961), with complications ranging from thrombosis (Spach *et al*., 1963) to blastic transformation (Fickers M *et al*., 1974). The term "congenital erythrocytosis" (CE), initially associated with the discovery of inappropriately high production of erythropoietin in familial cases (Whitcomb WH *et al*., 1980), is not entirely appropriate since the disease is rarely diagnosed at birth or in early infancy. However, CE is now the term most used to describe this entity, and for this reason CE will also be used in the present book.

The search for the molecular causes of CE began more than 45 years ago. The first germline mutation identified as causing CE was linked to abnormal hemoglobin affinity for oxygen (Charache *et al*., 1966; Lorkin PA, Lehmann H. 1970). Twenty years later, a new type of CE-causing

germline mutations concerning the receptor for erythropoietin was discovered (Yoshimura A *et al*., 1990). Subsequently, the first germline mutation associated with hereditary thrombocytosis (HT) was discovered in the gene coding thrombopoietin (Wiestner A *et al*., 1998). In the next decade, the rate of mutation discovery started to increase, and novel mutations associated with CE or HT were identified in several new genes, such as *VHL* (Ang SO *et al*., 2002) and *MPL* (the receptor for thrombopoietin) (Ding J *et al*., 2004). In the past 10 years, spectacular advances in sequencing techniques have greatly facilitated mutation discovery. Malignant forms of erythrocytosis (*eg*. polycythemia vera) and thrombocytosis (eg. essential thrombocythemia) were found associated with somatic mutations in 3 genes: mainly the V617F mutation in the *JAK2* gene (James C *et al*., 2005); mutations in the *CALR* (calreticulin) gene (Klampfl T *et al*., 2013); and more rarely, mutations in the *MPL* gene (Pikman Y *et al*., 2006). The latter finding led to a new pathogenic model stating that different defects in the *MPL* gene could lead to distinct forms of thrombocytosis, either benign in the case of germline mutation – in the context of HT – or malignant in the case of somatic mutation – in the context of essential thrombocythemia and primary myelofibrosis, both myeloproliferative neoplasms (MPNs) (Skoda RC, 2009). This model was confirmed by the discovery of new germline mutations in the *JAK2* gene in the context of HT (Mead A *et al*., 2012; Saint-Martin C *et al*., 2014).

Because of this rapid accumulation of new knowledge, in 2009 the European network MPN&MPNr-EuroNet (COST Action BM0902) was created, with the aim to facilitate and harmonize the molecular diagnosis of MPN and MPN-related (MPN-r) congenital diseases, particularly CE and HT. Whereas MPNs are the object of numerous studies, working groups, publications and guidelines, research teams who study CE and HT remain very few. In the meantime, the medical community has come to realize that cases of erythrocytosis and thrombocytosis caused by genetic defects are in fact not as rare as previously thought, and experts in these diseases are more and more solicited for advice.

The present book presents the latest knowledge on CE and HT, with the aim to help pediatricians, family practitioners and hematologists to diagnose CE and HT more easily. This book was

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edited on a voluntary basis. In spite of continued editing efforts, imperfections may still remain in terms of accurate and consistent definitions and terminology, text, referencing style, symbols, etc. Imperfections notwithstanding, the editors hope that this book will contribute to the diffusion of the knowledge on MPN-related disorders.

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I. CONGENITAL ERYTHROCYTOSIS

Preface

Erythrocytosis was first described 150 years ago by Denis Jourdanet, who found that the blood of people living at high altitude was more viscous than that of people living at sea level (Jourdanet, 1863). Forty-three years later Paul Carnot and his colleague C. de Flandre postulated that red cell production is regulated by a humoral erythropoietic factor which they called "Hemopoietine". They noticed that an increase of reticulocytes in normal rabbits occurred following the injection of blood plasma taken from anaemic donor rabbits that had earlier been subjected to a bleeding stimulus (Carnot and de Flandre, 1906). Later studies showed that this humoral factor, renamed erythropoietin (EPO), is elicited by tissue hypoxia. Advances in molecular biology led to the confirmation that hypoxia induces erythrocytosis due to increased EPO-mediated erythropoiesis and helped to establish the molecular mechanisms involved in the oxygen-sensing pathway (Wang and Semenza, 1996). Erythrocytosis can be secondary to a response to hypoxia, as in people living at high altitude or with cyanotic cardiac disease, or it can be congenital, due to alterations in the proteins involved in the sensing of oxygen, in the regulation of EPO/EPO receptor (EPOR) or in the hypoxia-sensing pathway (Semenza *et al*., 1991). Congenital erythrocytosis (CE) is a rare condition. Although presently eight genes are known to cause CE: *EPOR, EGLN1, EPAS1, VHL, HBB, HBA1, HBA2* and *BPGM*, for 70% of patients suspected of CE the causes of the disease are unknown, meaning there is no effective way to predict how the disease might evolve, and there is no curative treatment.

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Chapter 1
Erythrocytosis – A general overview

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The average adult produces approximately 2.4 million new red blood cells (RBC, also called erythrocytes) every second. These cells are rich in (Hb), an iron-containing protein that is responsible for the oxygen supply to the tissues. The red blood cell membrane is composed of proteins and lipids, and this structure provides properties essential for physiological cell function such as deformability and stability while traversing the circulatory system and specifically the capillary network. Mature red blood cells lack a cell nucleus and most organelles, in order to accommodate maximum space for Hb.

There are precise homeostatic mechanisms to ensure sufficient, but not excess, red blood cell production. Any imbalance in the homeostatic mechanisms can lead to excess of red blood cell production known as erythrocytosis.

Erythrocytosis can be either primary (due to an intrinsic defect of the erythroid compartment) or secondary (extrinsic to the red cell) and can be acquired or arise from hereditary alterations.

In general, primary erythrocytosis are characterized by an inappropriate response to low levels of erythropoietin (EPO) and secondary erythrocytosis have normal or high EPO levels.

The most frequent forms of erythrocytosis are acquired. Polycythaemia Vera (PV) is the most common type of acquired primary erythrocytosis with somatic mutations in the *Janus kinase* gene (*JAK2*) being responsible for almost 98% of the described cases (95% due to the c.1849G>T, p.Val617Phe mutation (V617F) in the exon 14, and 3% due to mutations in exon 12) (Cross, 2011). Acquired secondary erythrocytosis can develop from various diseases, such as cardiac, pulmonary or renal dysfunction, or external hypoxia due to smoking and carbon monoxide poisoning (reviewed by McMullin, 2008; Patnaik and Tefferi, 2009).

Hereditary or congenital erythrocytosis (CE), an uncommon group of inherited disorders, may either be primary or secondary to elevated EPO concentrations. The only known form of primary CE is associated with defects in the EPO receptor (EPOR), caused by mutations in the EPOR gene (*EPOR*), and is designated Primary Familial and Congenital Polycythemia (PFCP) (Huang *et al*., 2010) (Table 1).

Secondary CEs are generally characterized by normal or raised serum EPO. They can be a consequence of tissue hypoxia, being caused by congenital defects such as Hb variants with increased oxygen affinity due to mutations in the α – or β – globin genes (*HBB, HBA2, HBA1*) (reviewed by Wajcman and Galactéros, 1996 and by 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, namely mutations in the genes that encode the von Hippel-Lindau tumor suppressor (pVHL; gene VHL), the HIF-prolyl hydroxylase 2 (PHD2, gene EGLN1), and the hypoxia-inducible factor 2α (HIF-2α; gene EPAS1) (reviewed in Lee and Percy, 2011) (Table I.1).

Table I.1 – Congenital erythrocytosis – OMIM classification, genes and proteins associated.

Disease Group (OMIM number)	**Gene** (OMIM number)	**Location**	**Inheritance**	**Protein**	**Protein function**
ECYT1 (133100)	*EPOR* (133171)	19p13.2	Dominant (de *novo* cases described)	EPO receptor (EPOR)	- cognate receptor for EPO - activates JAK/STAT5 pathway - promotes survival, proliferation and differentiation of erythroid progenitor cells.
ECYT2 (263400)	*VHL* (608537)	3p25.3	Recessive (dominant cases described)	von Hippel Lindau (VHL)	- E3 ligase - involved in the ubiquitination and degradation of HIFα isoforms - targets HIFα for proteasomal degradation
ECYT3 (609820)	EGLN1 (606425)	1q42.1	Dominant	Prolyl hydroxylase domain-containing protein 2 (PHD2)	- oxygen sensor of HIF pathway - hydroxylates prolines in HIFα isoforms - requires O2, Fe, 2Og for activity/function.
ECYT 4 (611783)	EPAS1 (603349)	2p21	Dominant	Hypoxia Inducible Factor 2α (HIF2α)	- part of HIF2 transcription complex - hydroxylated by PHD2 - targeted to proteasome by VHL - controls EPO synthesis
High oxygen affinity: variant Hbs	HBB, HBA1, HBA2 (141900; 141800; 141850)	11p15.4; 16p13.3	Dominant	Hemoglobin (Hb)	- tetramer made up of two alpha and two beta chains - oxygen transport from the lung to the peripheral tissues
BPGM	BPGM (613896)	7q33	Recessive	Bisphosphoglycerate mutase (BPGM)	- regulation of hemoglobin's affinity for oxygen - controls the levels of 2,3-BPG

The elevated number of red blood cells and high haematocrit, with a consequent hyperviscosity, may result in CE patients presenting with symptoms and signs ranging from headaches, dizziness, epistaxis and exertional dyspnoea to pruritus after bathing. Moreover, thrombotic and haemorrhagic 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 haematocrit (Van Maerken, 2004).

1.1 Classification of Erythrocytosis

Erythrocytosis corresponds to an increase in the RBC count, Hb concentration and haematocrit above the reference range (adjusted to age, sex and living altitude), due to the expansion of the erythrocyte compartment in the peripheral blood.

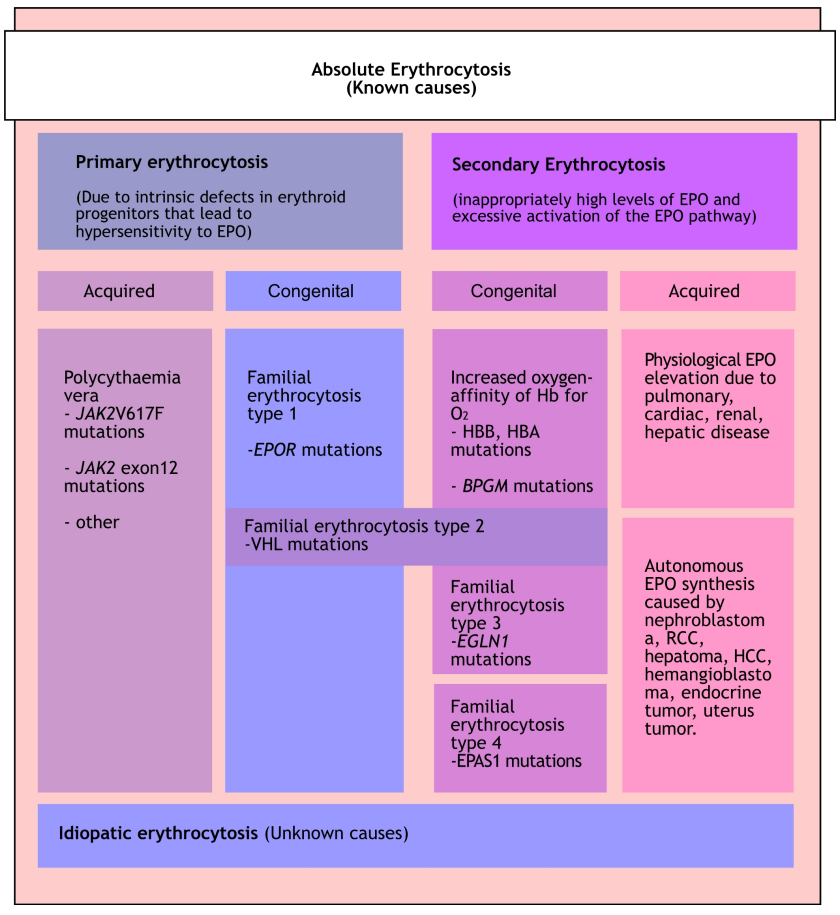
The term *Polycythemia* was also used but as it can be confused with *polycythemia vera*, a clonal stem cell disease, we avoid its use. However long-established terms, such as *Primary familial and congenital polycythemia* or *Chuvash polycythemia*, will be used.

Absolute erythrocytosis occurs when the red cell mass is greater than 125% of that predicted or when a raised Hb and haematocrit (Hb >18.5 g/dl or a haematocrit >52% in a male and a Hb > 16.5 g/dl or haematocrit >48% in the female) is observed in at least two separate blood counts performed at different time points.

Absolute erythrocytosis is distinct from relative erythrocytosis which is caused by a severe plasma volume reduction (*eg*, due to diuretics or severe diarrhea) and distinct from apparent erythrocytosis, caused by arterial hypoxaemia (*eg*, cigarette smoking, carbon monoxide poisoning or sleep apnoea) (Reviewed in McMullin *et al*., 2005).

Based on pathophysiology, absolute erythrocytosis can be classified either as primary or secondary erythrocytosis which can be of either congenital or acquired origin. Table I.2 summarizes the most frequent causes of absolute erythrocytosis.

Table I.2 – Classification of absolute erythrocytosis



1.1.1 Primary erythrocytosis

Primary erythrocytosis is a condition in which there is an intrinsic defect in the erythroid precursor cells. Primary erythrocytosis is usually characterized by low serum EPO levels and hypersensitivity of erythroid progenitors to EPO (Prchal, 1995; Kralovics and Prchal, 2000).

Acquired primary erythrocytosis is due to Polycythaemia Vera (PV), where erythroid progenitors carry a gain-of-function mutation in the *JAK2*, which leads to constitutive activation of the EPO signaling pathway at the EPOR level. Somatic *JAK2* V617F and *JAK2* exon 12 mutations are responsible for 98% of the cases described (Baxter *et al*., 2005; Scott *et al*., 2007; Cross, 2011).

The only molecularly characterized form of congenital primary erythrocytosis or familial erythrocytosis type 1 (ECYT1) is the autosomal dominant primary familial and congenital polycythemia (PFCP). PFCP, caused by gain-of-function mutations in the EPOR gene (*EPOR*), is a rare cause of erythrocytosis, (diagnosed in only 12% of the erythrocytosis with low serum EPO levels), no splenomegaly, normal Hb oxygen affinities and bone marrow erythroid progenitors that exhibit EPO hypersensitivity (Kralovics and Prchal, 2001; Huang *et al*., 2010).

1.1.2 Secondary erythrocytosis

Secondary erythrocytosis occurs when EPO production is increased for any reason, either as a physiological response to tissue hypoxia or in pathological circumstances (Robert and Means, 2004).

Most of the secondary erythrocytosis are acquired, where causes extrinsic to the erythroid compartment (for instance cardiac or pulmonary deficiency) induce hypoxia and, as a consequence, EPO is produced at higher levels and then drives the production of red blood cells.

Secondary CE can result from defects causing tissue hypoxia, as Hb variants with high oxygen affinity, due to mutations in the globin genes (*HBB* or *HBA*) (Charache *et al*., 1966, Robert and Means 2004, Rumi *et al*., 2009) or defective *BPGM* leading to 2,3 – BPG deficiency (Rosa *et al*. 1978, Lemarchande *et al*., 1992, Hoyer *et al*., 2004).

Secondary CE can also result from gain or loss of function mutations in the genes coding for some of the oxygen-sensing pathway proteins: VHL (von Hippel-Lindau), prolyl hydroxylase (*EGLN1*), and hypoxia-inducible factor (*EPAS1*) (Ang *et al*., 2002, Al-Sheikh *et al*., 2008, Gordeuk *et al*., 2005, Ladroue *et al*., 2008, Martini *et al*., 2008, Percy *et al*., 2007, Percy *et al*., 2008a, Percy *et al*., 2006).

1.1.3 Idiopathic erythrocytosis

Erythrocytosis in patients with no identified causes is designated Idiopathic erythrocytosis (IE). In current practice the etiology of 70% of IE cases remains unknown, meaning that no proper diagnosis can be made, no prognosis or advice can be provided to patients and their family, and no curative treatment exists.

1.2 Molecular etiology of Congenital Erythrocytosis

Presently, eight genes are known to be associated with CE: *EPOR, EGLN1, EPAS1, VHL, HBB, HBA1, HBA2* and *BPGM* (Figure below). The characteristics of these genes, the type of mutations, phenotype associated, mode of transmission and number of cases described are presented in Tables I.1 and I.3.

As mutations in these genes were identified only in 30 – 40% of all known CE patients it can be anticipated that additional genes should be implicated.

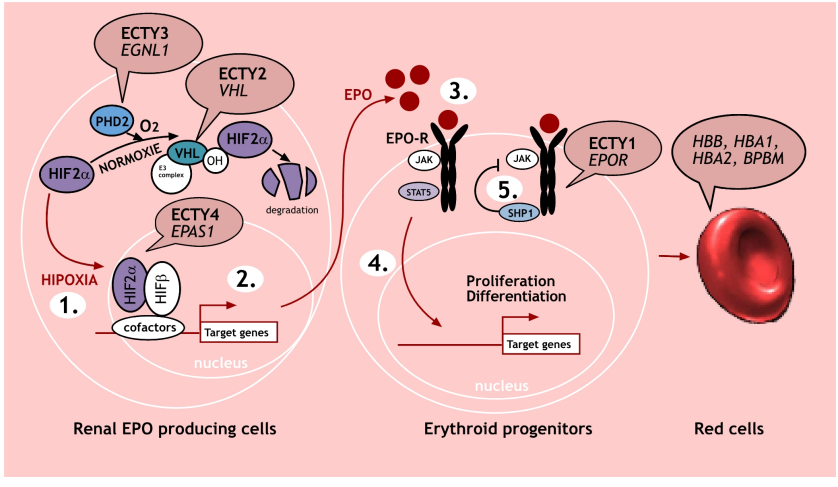


Figure I.1 Schematic pathway involved in erythrocytosis and mutated genes associated with the different types of erythrocytosis. The germline mutations target different actors involved in red cell production (Figure I.1).

The mutated genes are represented in a speech ballon and the type of erythrocytosis (ECYT) is indicated for each gene. The red cell production pathway is represented as follow: in presence of oxygen (normoxia), the α-subunit of the hypoxia inducible factor (HIF) is hydroxylated and binds the VHL protein that induces its ubiquitination and degradation in the proteasome. 1. In the absence of oxygen (hypoxia), HIF-2α is stabilized, translocates into the nucleus and associates to HIF-1β2. The active HIF transcription factor induces expression of a numbers of target genes, including erythropoietin (EPO). 3. EPO is released in the circulation from renal EPO producing cells. 4. EPO binds its receptor, EPOR, on the surface of erythroid progenitors, in the bone marrow. 5. This binding induces a cascade of phosphorylation and signal transduction leading to proliferation and differentiation of the progenitors towards mature red cells. Genetic alteration of genes encoding some of these actors induces overproduction of red cells and erythrocytosis. JAK2: Janus Kinase 2; SHP1: Src homology 2 domain-containing phosphatase-1 region; STAT5: signal transducer and activator of transcription factor 5; HBB, Hb -β; HBA1, Hb-α1; HBA2, Hb -α2; BPBM, bisphosphoglycerate mutase.

Table I.3 – Genes associated with Congenital Erythrocytosis

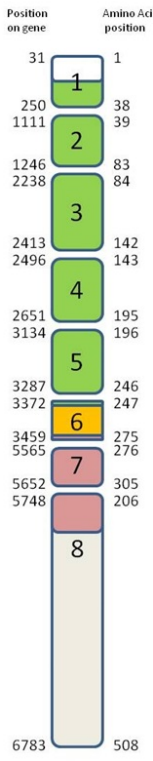
Gene	Type of mutation	Protein Mutated	N° of mutations described	Effect on protein	N° of cases described in the literature
HBB	Missense	β-globin chains	>100 variants	Increases Hb affinity for O2	Not known
HBA1 and *HBA2*	Missense	α-globin chains	>30	Increases Hb affinity for O2	Not known
BPGM	Missense	2,3 -BPG	3	Reduced synthesis of 2,3-BPG	5 cases (2 families)
EPOR	Nonsense / Missense / Small deletions	EPOR	23	Loss of the negative regulatory domain	114 cases (22 families)
VHL	Missense / Nonsense	VHL	18	Loss-of-function	55 cases (30 families) (73 cases in Chuvash)
EGLN1	Missense / nonsense / Small deletions or insertions	PHD2	19	Loss-of-function	21 cases (17 families)
EPAS1	Missense	HIF2-α	10	Gain-of-function	22 cases (12 families)

1.2.1 Congenital Erythrocytosis Type 1 (ECYT 1)

The first mutation in the *EPOR* gene was found in a Finnish man and his 29 family members, as described by de la Chapelle *et al*. (1993). Since then several mutations have been found, in the heterozygous state, all of it located in exon 8, which encodes the C-terminal negative regulatory domain of the protein (Fig. I.2). These mutations induce a gain-of-function and result in Primary Congenital Familial Erythrocytosis (PFCP), which is also known as familial erythrocytosis type 1 (ECYT1; OMIM 133100; Table I.1).

Whereas the majority of adult patients with *EPOR* mutations had only mild symptoms, some cases were reported to present with severe and even fatal clinical complications such as arterial hypertension, intracerebral haemorrhage, deep vein thrombosis, coronary disease and myocardial infarction (Prchal *et al*, 1995; Sokol *et al*, 1995; Kralovics *et al*, 1997a; Kralovics *et al*, 1998;). In the majority of patients no regular treatment is needed, however, hypertension should be treated and phlebotomy used, either when patients present with hyperviscosity symptoms or regularly performed aimed to maintain the haematocrit at an almost normal level.

A.



B.

EPOR Functional domains	Amino acid position	Variants in the *EPOR* gene associated with ECYT1		
		cDNA mutation	Predicted amino acid change	References
Signal peptide	1 - 24			
Extracellular	25-250			
Transmembrane	251-273			
Juxtamembrane	274-338			
Cytoplasmic	339-508			
		c.1141_1142del	p.Pro381Glnfs*2	Al-Sheikh et al., 2008
Tyr residues:		c.1195G>T	p.Glu399*	Arcasoy et al., 2002
Interaction with STAT5 and activation	368	c.1234delT	p.Ser412Argfs*41	O'Rourke et al., 2011
	386	c.1249G>T	p.Glu417*	Perrotta et al., 2010
	426	c.1252_1255del	p.Gly418Profs*34	Petersen et al., 2004
Required for SPH1 and SOCS3 binding inactivation	454	c.1271_1272del	p.Phe424*	Al-Sheikh et al., 2008
	456	c.1273G>T	p.Glu425*	Kralovics et al., 2001
	468	c.1278C>G	p.Tyr426*	Kralovics et al., 1998
	485	c.1281dupT	p.Ile428Tyrfs*17	Kralovics et al., 1997
	489	c.1285del	p.Leu429Trpfs*24	Al-Sheikh et al., 2008
	504	c.1288dupG	p.Asp430Glyfs*15	Sokol et al., 1995
		c.1300C>T	p.Gln434*	Furukawa et al., 1997
		c.1299_1305del	p.Gln434Cysfs*17	Arcasoy et al., 1997
		c.1316G>A	p.Trp439*	la Chapelle et al., 1993
		c.1317G>A	p.Trp439*	Rives et al., 2007
		c.1460A>G	p.Asn487Ser	Le Couedic et al., 1996
		c.1462C>T	p.(Pro488Ser)	Sokol et al., 1994

Figure I.2. A – Schematic representation of the *EPOR* gene. Exons (1 – 8) represented in colored boxes (not in scale). B. Some of the mutations already described in the *EPOR* gene, aminoacid position and functional domains of the protein.

1.2.2 Congenital Erythrocytosis Type 2 (ECYT 2)

VHL gene

The *VHL* gene (MIM 608537) is located on chromosome 3 (locus 3p25.3) and spans 10kb. 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 (pVHL19), both are functionally active (Iliopoulos, O, 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.

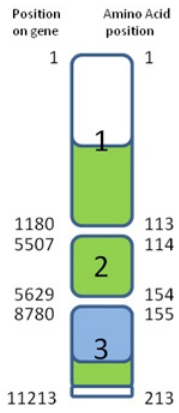
The first loss-of-function mutation in the *VHL* gene associated with CE was found in the Chuvash autonomous republic of Russia where polycythaemia is an endemic disorder. Chuvash polycythemia was found to be caused by a homozygous c.598C>T, p.Arg200Trp (R200W) *VHL* mutation (Ang *et al*., 2002a, 2002b) that has arisen from a single founding or single mutation event occurring 14,000 to 50,000 years ago (Lui *et al*, 2004).

Later, the *VHL*-R200W mutation was also observed in non-Chuvash patients and other VHL mutations, in the homozygous or compound heterozygous state, were described in association with familial erythrocytosis type 2 (ECYT2; OMIM 263400; Table I.1) (Fig. I.3).

Although CE type 2 is considered a recessive disease, the occurrence of individuals heterozygous for *VHL* mutations with erythrocytosis has been described.

Clinical features of Chuvash polycythaemia include rubor, vertebral haemangiomas, varicose veins, low blood pressure, cerebral vascular events, and peripheral thrombosis. (Gordeuk *et al*., 2004).

A.



B.

VHL Functional domains	Amino acid position	Variants in the *VHL* gene associated with ECYT2		
		Amino acid change	cDNA mutation	References
β-domain: Interaction with HIF2α	63-154 / 65 – 117	p.Arg79Cys	c.235 C>T	Bento et al., 2005
		p.Gly104Val	c.311G>T	Cario et al., 2005
		p.Asp126Asn	c.376G>A	Bond et al., 2011
		p.Asp126Tyr	c.376G>T	Pastore et al., 2003a
		p.Val130Leu	c.388G>C	Pastore et al., 2003a
		p.Pro138Leu	c.413C>T	Lanikova et al., 2013
		p.Gly144Arg	c.430 G>A	Randi et al., 2005
α-domain Interaction with Elongin BC complex	155 - 192	p.Tyr175Cys	c.524A>G	Bento et al., 2005
		p.Ser183Leu	c.548C>T	Bond et al., 2011
		p.Leu188Val	c.562 C-G	Pastore et al., 2003b
		p.His191Asp	c.571 C-T	Pastore et al., 2003b
		p.Pro192Thr	c.574C>A	Percy et al., 2007
		p.Pro192Ser	c.574 C-T	Pastore et al., 2003b
β-domain Interaction with HIF2α	193-204	p.Arg200Trp	c.598 C-T	Ang et al., 2002

Figure I.3. A – Schematic representation of the *VHL* gene. Exons (1 – 3) represented in coloured boxes (not in scale). B. Some of the mutations already described in the *VHL* gene, amino-acid position and functional domains of the protein.

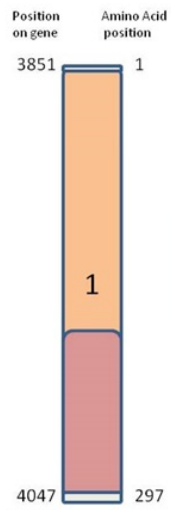
1.2.3 Congenital Erythrocytosis Type 3 (ECYT 3)

EGLN1 gene

There are three PHD isoenzymes (PHD1, PHD2 and PHD3), but PHD2 was found to be the key enzyme in catalyzing the prolyl hydroxylation of HIFα, using oxygen as a co-substrate (Kunz and Ibrahim, 2003; Berra *et al*., 2003; Percy 2006). PHD2 is encoded by the *EGLN1* gene (OMIM 606425), which is located on chromosome 1q42.1, and comprises 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 (ECYT3; OMIM 609820) (Table I.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 of one family (3 family members). The mutation resulted in a p.Pro317Arg (P317R) substitution in a highly conserved region of the protein (Fig. I.4).

A.



B.

PHD2 Functional domains	Amino acid position	Variants in the *EGLN1* gene associated with ECYT3		
		Amino acid change	cDNA mutation	References
Egl nine homolog 1 - Mature protein	2 - 426			
Catalytic domain	181 - 426	p.Pro200Gln	c.599C>A	Ladroue et al., 2012
		p.Met202Ilefs*72	c.606delG	Al-Sheikh et al., 2008
		p.Asn203Lys	c.609C>G	Albiero et al., 2012
		p.Asp254His	c.760G>C	Ladroue et al., 2012
		p.Arg281Thrfs*4	c.840dupA	Al-Sheikh et al., 2008
2OG Fe(II) dioxygenase:	291 - 392	p.Lys291Ile	c.872A>T	Albiero et al., 2012
		p.Pro317Arg	c.950C>G	Percy et al., 2006
Iron binding	313			
Iron binding	315	p.Arg371His	c.1112G>A	Percy et al., 2007
Iron binding	374	p.His374Arg	c.1121A>G	Ladroue et al., 2008
2-oxoglutarate binding	383	p.Gln377*	c.1129C>T	Al-Sheikh et al., 2008
		p.Arg398*	c.1192C>T	Ladroue et al., 2012
		p.Lys423Glu	c.1267A>G	Albiero et al., 2012

Figure I.4 – A. Schematic representation of the *EGLN1* gene. Exons (1 – 5) represented in colored boxes (not in scale). B. Some of the mutations already described in the *EGLN1* gene, amino-acid position and functional domains of the protein.

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 deregulation of EPO production. Since then more than 22 patients were found to carry mutations in this gene, all of them in the heterozygous state (Table I.3).

1.2.4 Congenital Erythrocytosis Type 4 (ECYT4)

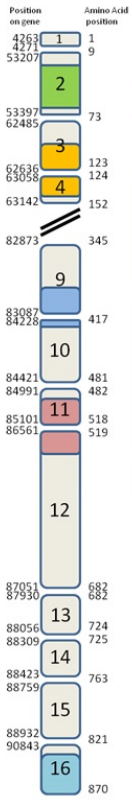
EPAS1 gene

The HIF transcription factor has three isoforms, HIF1α, HIF2α and HIF3α. HIF1α was first identified as a mediator of EPO induction in response to hypoxia in vitro (Wang and Semenza, 1996), however HIF-2α was later confirmed as the primary transcription factor that induces EPO expression (Scortegagna, 2003; Warnecke, 2004; Hickey 2007, Percy 2008a). The degradation of HIF2α occurs via the hydroxylation of the residues Pro 405 and Pro 531.

The *EPAS1* gene (OMIM 603349), which encodes the transcription factor HIF2α, is located on chromosome 2p21, contains 16 exons and spans at least 120 kb. From the 5160 bp long *EPAS1* mRNA is translated 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 (ECYT4, OMIM 611783), showing autosomal-dominant inheritance. 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) (Fig. I.5).

A.



B.

Hif-2α Functional domains	Amino acid position	Variants in the *EPAS1* gene associated with ECYT4		
		Amino acid change	cDNA mutation	References
Basic helix– loop–helix （bHLH)	21 – 68			
Period-ARNT-Single-minded （PAS)	97 - 147			
Oxygen-degradation domains （ODD)	390-423	p.Phe374Tyr	c.1121T>A	Lorenzo et al., 2012
Secondary Hydroxylation residue by PHD	405			
N-terminal activation domain (NAD)	496-542	p.Pro534Leu	c.1601C>T	Percy et al., 2008
		p.Met535Ile	c.1605G>A	Martini et al., 2008
		p.Met535Thr	c.1604T>C	Percy et al., 2012
		p.Met535Val	c.1603A>G	Percy et al., 2008
Primary Hydroxylation residue by PHD	531	p.Gly537Arg	c.1609G>A	Percy et al., 2008
		p.Gly537Trp	c.1609G>T	Percy et al., 2008
		p.Asp539Glu	c.1617C>G	van Wijk et al., 2010
		p.Phe540Leu	c.1620C>G	Percy et al., 2012
C-terminal activation domain (CAD)	830-870			
Hydroxylation residues by the factor inhibiting HIF (FIH)	847			

Figure I.5 – A. Schematic representation of the *EPAS1* gene. Exons (1 – 16) represented in colored boxes (not in scale). B. Some of the mutations already described in the *EPAS1* gene, amino-acid position and functional domains of the protein.

1.2.5 Hemoglobin with high oxygen affinity

In a normal adult almost all of the oxygen in the blood is carried by haemoglobin A (HbA), a globular protein composed of two alpha globin chains, two beta globin chains and four heme groups each one with a porphyrin ring with an iron (Fe) atom in its center (Fig. I.5). Normally, the Fe is in the ferrous state (Fe^{+2}) and can bind oxygen reversibly.

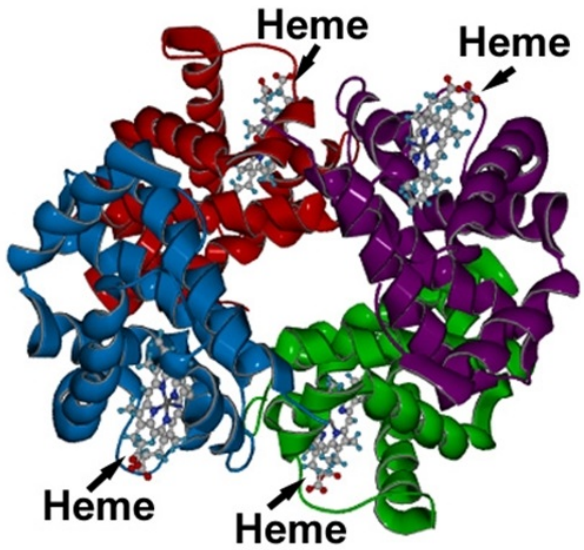


Figure I.6 – Ribbon Structure of the Hb molecule. Each of the 4 globin chains is represented in a different colour. The heme molecule is shown in white.

The Hb tetramer exists in equilibrium between two quaternary conformations: oxygenated or "relaxed" (R) and deoxygenated or "tense" (T). The T form has a lower affinity for oxygen than the R form. At some point during the sequential addition of oxygen to the four hems, transition from the T to R configuration occurs and the oxygen affinity of Hb increases dramatically.

The oxygen dissociation curve, which reflects these changes, can be modified in several ways. First, oxygen affinity is decreased with increasing carbon dioxide (CO_2) tensions (the Bohr effect). This facilitates oxygen loading to the tissues, where a drop in pH due to CO_2 influx lowers oxygen affinity. In contrast, in the lungs, efflux of CO_2 and an increase in intracellular pH increases oxygen affinity. Oxygen affinity is also modified by the level of 2,3-BPG in the RBC. Increasing concentrations shift the oxygen dissociation curve to the right reducing oxygen affinity, whereas diminishing concentrations have the opposite effect. The oxygen affinity is measured by the value of P_{50}, the partial pressure of oxygen at which Hb is half saturated (Bunn and Forget, 1986).

HBB and *HBA* genes

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

The *HBB* gene (OMIM 141900) is also comprised of three exons with a transcript of 754 bp long and 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 Hb of 199 g/L who was seen at the haematology clinic in Johns Hopkins Hospital by Samuel Charache (Charache *et al*., 1966). A thorough family study revealed 15 other members with increased Hb levels, all of them showing an abnormal Hb band on electrophoresis. In addition, the oxygen

dissociation curve was significantly displaced to the left, indicating increased oxygen affinity. Structural analysis established that this was an alpha-chain variant with a substitution of leucine for arginine at position 92 and the hemoglobin variant was subsequently called Hb Chesapeake.

Since then more than 100 mutations have been described in the globin genes, the majority at the *HBB* locus, causing high oxygen affinity Hb variants. Erythrocytosis associated to high oxygen affinity Hb variants is 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 Hb function 1) the $\alpha 1 \beta 2$ 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 the database, Hb Var (http://globin.bx.psu.edu/hbvar/menu.html). As only patients with new mutations are registered, it is not possible to estimate the real incidence and prevalence of the high affinity Hb variants.

CE patients with high affinity Hbs are generally asymptomatic, may have facial and mucosal high colour, hyperviscosity symptoms and thromboembolic episodes related to the high haematocrit (Fairbanks *et al*., 1971; Weatherall *et al*, 1977).

BPGM gene

The *BPGM* gene (OMIM 613896; locus 7q33) extends over 22 kb and contains 2 exons, encoding a protein of 259 amino acids (MW~30 kDa).

The BPGM enzyme is important in the regulation of hemoglobin's affinity for oxygen by controlling the level of 2,3-BPG, which is generated during glycolysis. When 2,3-BPG is bound to Hb it decreases the affinity of Hb for oxygen. Consequently, it allows efficient delivery of oxygen to tissues. 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).

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1.3 Epidemiology

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Erythrocytosis presents with an increased red cell mass. The myeloproliferative diseases are rare neoplasms where there is an increased production of red cells and the myeloproliferative neoplasm polycythaemia vera and secondary acquired causes such as respiratory disease are the most usual reasons for an erythrocytosis. Congenital causes constitute a very rare subset of those with an increased red cell mass compared to all the more common acquired reasons.

There is no information on incidence and prevalence of congenital erythrocytosis or these excessively rare disorders which have been mostly reported as individual cases. A combined annual incidence of polycythaemia vera has been calculated at 0.84 per 100,000 (Titmarsh, *et al*, 2014). This can only be calculated on studies prior to 2005 before the *JAK2* mutation was part of the diagnostic criteria. Many of the studies on incidence included in the meta-analysis of incidence may include cases which have erythrocytosis only in an era when diagnostic criteria were not as definitive and some cases on congenital erythrocytosis may be included in older case series of myeloproliferative disorders. Such cases, if congenital, would constitute a very small proportion of the overall cases. Overall, the incidence of congenital erythrocytosis is unknown but much lower than that of the myeloproliferative neoplasm polycythaemia vera.

Prevalence rates for polycythaemia vera were reported in few studies and there were insufficient studies to do a meta-analysis (Titmarsh *et al*, 2014) may become available. As regards, congenital erythrocytosis there is therefore no information on prevalence. As databases on these disorders prevalence information may be able to be developed (Bento *et al*, 2014)

The epidemiology of the myeloproliferative disorders has been reviewed systematically and from the published literature a variety of diseases, occupations, and exposures have been associa-

ted with these acquired disorders (Anderson *et al*, 2012). For the very rare congenital erythrocytosis there is no epidemiological information.

These are germ-line inherited events and the cause of the mutation event is not known. However, In the case of the VHL Chuvash mutation it has been shown that it has arisen from a single founding or single mutation event 14,000 to 50,000 years ago (Lui *et al*, 2004). It is likely that other mutations may have arisen many years ago and from evolutionary principles acquisition of such mutations would be expected to give a survival advantage hence allowing the mutations to continue.

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Chapter 2
Hypoxia pathway

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2.1 Oxygen sensing

Accurate oxygen homeostasis regulation is essential for the survival of all organisms. Oxygen sensing is a fundamental biological process regulated by enzymes belonging to the 2-oxoglutarate-dependent oxygenase (dioxygenase) superfamily. Indeed, there are currently 60 known dioxygenases that include the prolyl hydroxylase domain (PHD) enzymes and Factor Inhibiting HIF (FIH), which induce the hydroxylation and degradation of the Hypoxia inducible factor (HIF), the Procollagen-proline dioxygenase, and dioxygenase involved in epigenetic modification (TET, JMJ) (Fig. I.7).

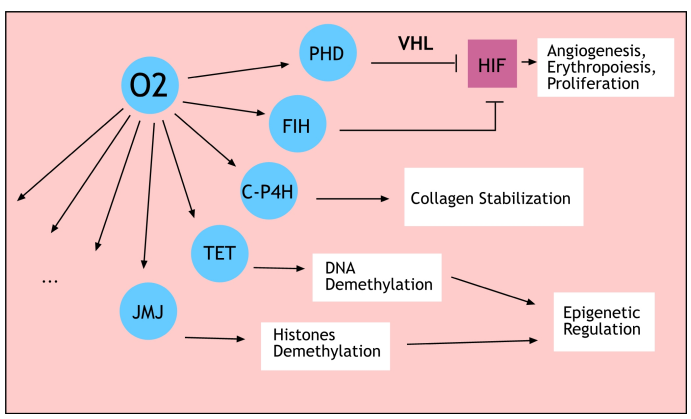

Figure I.7 Oxygen is a crucial co-factor of many enzymes that include Prolyl hydroxylase domain (PHD) enzymes, Factor Inhibiting HIF (FIH), involved in the degradation and inhibition of the Hypoxia Inducible Factor (HIF); C-P4H (Procollagen- Prolyl 4-hydroxylase), involved in hydroxylation of prolyl residues in preprocollagen, which induce the stabilisation of collagen; TET proteins, involved in convertion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), which induce demethylation of DNA and JMG (jumanji) family of histone demethylases.

All the dioxygenases share a common biochemical reaction They catalyse the oxidation of an organic substrate using a dioxygen molecule, mostly by using ferrous iron Fe(2+) as the active site cofactor and 2-oxoglutarate (2OG) as a co-substrate. During the catalytic cycle, the enzyme forms a reactive oxidizing species (Fe[IV]=O) which oxidizes its substrate(s) to give the hydroxylated product(s) and 2OG is decarboxylated to succinate and CO2 (Fig. I.8). As a negative feedback control, succinate (a competitor of 2OG) inhibits the hydroxylation reaction.

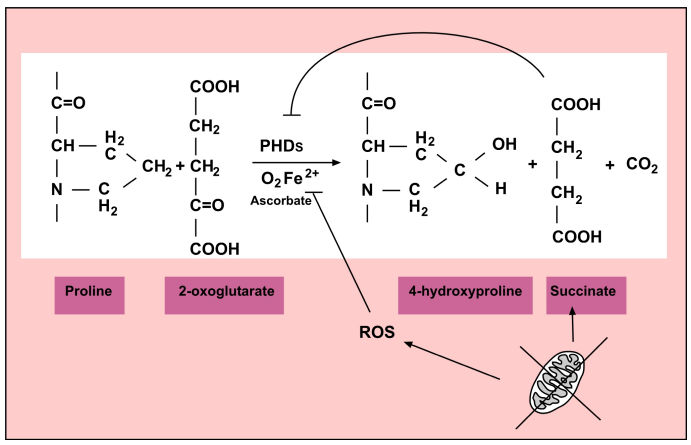

Figure I.8 Hydroxylation reaction of HIF1α prolines by PHD dioxygenases. ROS: Reactive Oxygen Species.

The diminution of oxygen concentration (hypoxia) leads to the inactivation of dioxygenase and arrest of hydroxylation reactions. Dioxygenase can also be inactivated by other pathways named "pseudo-hypoxic" pathways. For example, mutations in the Krebs cycle enzymes fumarate hydratase (FH) and succinate dehydrogenase (SDH) activate HIF. In these cases, HIF activation could be a consequence of inhibition of the PHD by accumulated fumarate/or succinate, which are potential 2-oxoglutarate analogs and competitors. Mutations in isocitrate dehydrogenase can lead to substantially increased levels of 2-hydroxyglutarate, which has the potential to inhibit all of the dioxygenase (Xu *et al*., 2011). These inhibitions may have a major impact on pathology. Germline mutations in *FH* can predispose to the development of papillary type II renal cancers and leiomyomas while germline mutations in *SDH* predispose to hereditary pheochromocytoma and paraganglioma (extra-adrenal pheochromocytoma) syndrome and renal cancers. Furthermore, mutations in *IDH* are associated with haematological malignancies and brain tumour development.

2.2 Regulation of Hypoxia Inducible factors

The transcription factor Hypoxia Inducible Factor (HIF), which is conserved during evolution from basal metazoans to primates, plays an essential role in oxygen homeostasis during embryonic development and postnatal life. Under limiting O2 (hypoxic) conditions, HIF upregulates the expression of a number of target genes including that encoding for the glycoprotein hormone erythropoietin (EPO), which regulates the proliferation and differentiation of erythroid progenitors, thereby linking decreased tissue oxygenation to an adequate erythropoietic response.

HIF is an α/β hetero-dimer consisting of a tightly regulated oxygen-labile β-subunit and a constitutive β-subunit. Three HIF-α isoforms exist: HIF-1α and HIF-2α, which are closely related and extensively studied, and HIF-3α, a more distantly related isoform that possibly plays an antagonistic role in the regulation of the other isoforms (Fig. I.9).

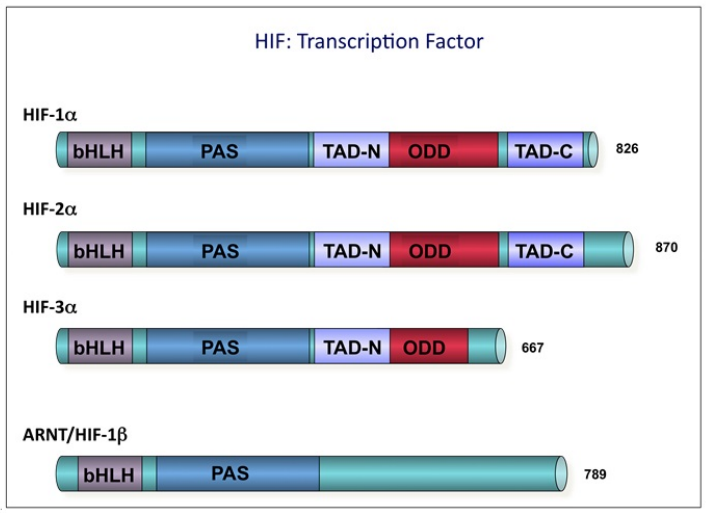

Figure I.9 Structure HIF subunits. bHLH: basic Helix Loop Helix, PAS: PER-ARNT-SIM, TAD: TransActivation domain, ODD: oxygen Degradation Domain.

The HIF-α subunits contain an oxygen-dependent degradation (ODD) domain which confers oxygen-dependent instability and two independent transcriptional activation domains (HIF-1α N-TAD: amino acids 531-575) and (C-TAD: amino acids 786-826). Hydroxylation is the main post-translational modification regulating the oxygen dependent stability of HIF-α subunits. In humans, HIF-α hydroxylation is catalyzed by three prolyl hydroxylases (PHD 1-3) and an asparaginyl hydroxylase, Factor inhibiting HIF (FIH), (collectively called the HIF hydroxylases). The HIF hydroxylases are dioxygenases, which utilize 2-oxoglutatrate (2-OG) and oxygen as co-substrates, providing a molecular basis for the oxygen-sensing function of these enzymes (Berra *et al*., 2006)

(Kaelin and Ratcliffe, 2008). The PHD proteins control the stability of HIF-α subunits, and FIH regulates their transcriptional activity. PHDs hydroxylate prolyl residues located within the HIF-α ODD (P402 and P564 for HIF-1α; P405 and P531 for HIF-2 α), which allows binding of HIF-α to the von Hippel-Lindau (VHL) protein, a recognition component of an E3 ubiquitin ligase multiprotein complex. Binding of VHL to HIF-α isoforms induces ubiquitination which targets them for degradation by the proteasome (Fig. I.1). Of the three PHD isoforms (1-3), in mammalian cells, PHD2 appears to be a particularly critical oxygen sensor controlling HIF-1α stability *in vivo* (Appelhoff *et al*., 2004, Berra *et al*., 2003, Kaelin and Ratcliffe, 2008). FIH hydroxylates the asparagine in the C-TAD (Asn 803/HIF-1α, Asn 847/ HIF-2α), a modification which inhibits the binding of HIF-α to the p300/CBP transcriptional co-activator proteins (Lando *et al*., 2002).

Under hypoxic conditions, when the co-factor oxygen is limiting, hydroxylation of HIF-α subunits slows down resulting in HIF-α stabilization. HIF-α then translocates to the nucleus, associates with the HIF-1β subunit and forms an active heterodimer. Upon recruiting appropriate co-activators such as p300/CBP, the HIF-α/β heterodimer binds to a hypoxia response elements (HREs, pentanucleotide sequence 3'-(A/G)CGTG-5') within DNA and activates expression of HIF target genes (Wenger and Hoogewijs, 2010, Wenger *et al*., 2005). More than 200 genes are transcriptionally regulated by HIF and many genes are involved in many pathways such as cell proliferation and survival, angiogenesis [vascular endothelial growth factor (VEGF) and platelet derivative growth factor (PDGFβ)], cell proliferation [transforming growth factor alpha (TGFα)], regulation of glucose uptake and metabolism [glucose transporter (Glut-1)], autophagy (BNIP3), cell cycle regulation (Cyclin D1) and erythropoiesis (via the synthesis of EPO (Semenza *et al*., 1991)) (Fig. I.10).

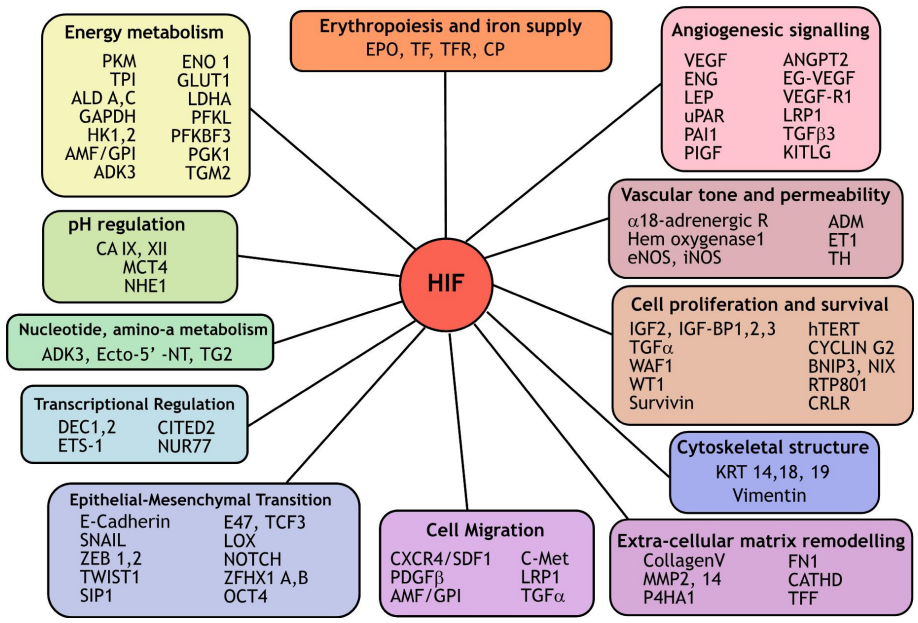

Figure I.10 HIF target genes (from (Chowdhury *et al*., 2008, Keith *et al*., 2012, Semenza, 2003, Wenger *et al*., 2005)

The regulation of HIF target genes is very complex and not yet fully understood. It is dependent on the cell type, the specific HIF-α subunit, the N- or C-terminal transactivation domain involved (Pugh *et al*., 1997), and the oxygen concentration in addition to other context dependent factors. Moreover, PHD and FIH have distinct and different affinities for oxygen as reflected by their Km values for oxygen. PHDs have a lower affinity and hence are inhibited more rapidly than FIH when the concentration of oxygen decreases (all other factors being equal). Therefore, under moderate hypoxic conditions, the HIF–α subunits are stabilized because PHDs are inhibited but

the C-TAD is still inhibited as FIH is active. Consequently, only genes regulated by the N-terminal transactivation domain are induced (Dayan *et al*., 2006). Furthermore, PHDs have been shown to manifest a certain degree of selectivity between HIF-1-α and HIF-2-α (Appelhoff *et al*., 2004).

The HIF-1-α isoform was first identified by Wang and collaborators from hypoxia-induced binding of a transcription factor complex to the EPO enhancer (Wang *et al*., 1995). However, it was considered to be the major HIF–α isoform involved in the regulation of EPO is HIF-2-α, in mice (Kim *et al*., 2006, Rankin *et al*., 2007, Scortegagna *et al*., 2005) and in human (Percy *et al*., 2008). HIF-2-α also regulates genes required for cell survival under low oxygen tension, such as heme synthesis (ALAS2), globin chains production (GATA1) and iron regulation (TRF2, TF) (Haase, 2010, Lok and Ponka, 1999, Zhang *et al*., 2011, Zhang *et al*., 2012). Recently, HIF-3-α has also been considered as a potential isoform which may be able to regulate transactivation of genes. The human HIF-3-α locus is subject to extensive alternative splicing, leading to multiple variants. Studying these variants established them as inhibitors of HIF1-α and HIF-2-α activity. However, depending on the isoform and experimental conditions (HIF-1β is not a limiting factor), HIF-3-α regulates a distinct transcriptional response to hypoxia (Zhang *et al*., 2014), including EPO production (Heikkila *et al*., 2011).

The major source of EPO production is the interstitial cells in the kidney (Bussolati *et al*., 2013, Lacombe *et al*., 1988, Maxwell *et al*., 1993, Paliege *et al*., 2010). EPO is then released in the circulation and binds its receptor, EPOR, on the surface of erythroid progenitors in the bone marrow. Once EPO binds its cognate receptor there is initiation of an intra-cellular signalling cascade via JAK2 (Janus Kinase 2) and STAT5 (signal transducer and activator of transcription factor 5). STAT5 homodimerises and translocates into the nucleus where it activates the transcription of various genes involved in inhibition of apoptosis and growth and differentiation of erythroid progenitors, thereby adjusting red blood cell mass to oxygen delivery requirements.

The effect of the hypoxia pathway on erythroid progenitor proliferation is therefore indirect, via the EPO production. However, increasingly more data has been accumulated in favour of an EPO-independent effect of hypoxia pathway on erythropoiesis. For example, deletion of *Phd2*

targeted in haematopoietic precursors induces erythrocytosis, and bone marrow cells derived from these as well as those from an induced globally-deleted *Phd2* mouse line display hypersensitivity to EPO, as assessed by Erythroid Burst Forming Unit (BFU) assays (Arsenault *et al*., 2013, Li *et al*., 2010). Moreover, loss of *Phd2* in haematopoietic stem cells (HSC) induced by either a CD68-driven or globally inducible cre transgene results in increased numbers of Lineage- Sca1+ c-Kit+ (LSK) cells, which are early HSCs, and subsets of these cells, which include MPP1, MPP2, and MPP3 multipotent progenitors (Singh *et al*., 2013, Takeda *et al*., 2008). In addition, granulocytes and erythroid progenitors from BFU assays of patients with mutation in the hypoxia sensing pathway express elevated level of RUNX1 (Kapralova *et al*., 2014). RUNX1, a potential HIF target gene, is essential for the regulation of erythroid differentiation and maturation and expression of globin genes.

2.3 Genes of the hypoxia pathway involved in erythrocytosis

In the context of hereditary secondary erythrocytosis, several mutations in three genes of the oxygen-sensing pathway have been described: PHD2 (encoded by *EGLN1*), HIF-2α (encoded by *EPAS1*) and von Hippel-Lindau (encoded by *VHL*) (Bento *et al*., 2014). Interestingly, more recently, a germline mutation in PHD1 (encoded by *EGLN2*), c.188T>A, p.Ser61Arg, has been identified in a patient with erythrocytosis associated with pheochromocytoma and paraganglioma (Yang *et al*., 2015). The authors demonstrated the loss of wild type *EGLN2* allele in the tumours, suggesting a role of PHD1 mutant in tumour occurrence.

– *VHL* gene mutations:

Congenital erythrocytosis linked to a defect in the hypoxia sensing pathway was first described in Russian patients from the Chuvash region (Ang *et al*., 2002). These patients are homozygotes for the c.598C>T, p.Arg200Trp *VHL* (R200W) mutation and present with a congenital erythrocytosis named Chuvash polycythaemia. Clinical features of Chuvash polycythaemia include rubour,

vertebral haemangiomas, varicose veins, low blood pressure, cerebral vascular events, and peripheral thrombosis. Patients suffer from iron deficiency secondary to phlebotomy, and elevated pulmonary arterial pressure (PAH) (Bond *et al*., 2011, Gordeuk *et al*., 2004, Sable *et al*., 2012). Survival in the Chuvash patients was found to be reduced compared to control groups due to higher rates of arterial and venous thromboses, and haemorrhagic events (Gordeuk *et al*., 2004). This specific R200W mutation has also been identified in combination with other *VHL* mutations (compound heterozygosity) and, since then, other missenses *VHL* mutations have been described, also in homozygous status (Bento *et al*., 2014). One mutation (*VHL*-D126N) is particularly associated with severe PAH (Bond *et al*., 2011, Sarangi *et al*., 2014).

It is known that the HIF signalling is involved in Chuvash polycythaemia (Ang *et al*., 2002, Hickey *et al*., 2007, Kapralova *et al*., 2014, Rathmell *et al*., 2004) but the exact mechanisms which may explain the heterogeneity of the clinical phenotype (differences in serum EPO level and propensity to develop thrombosis and PAH) is still unexplained. Recently, it has been shown that pVHL also forms a heterodimeric E3 ligase complex with SOCS1 (suppressor of cytokine signalling 1) to target phosphorylated (p)JAK2 for ubiquitin-mediated degradation (Russell et al., 2011). R200W mutants have altered affinity for SOCS1 and fail to degrade pJAK2. This may explain the EPO hypersensitivity of red blood cell progenitors in Chuvash patients (Ang *et al*., 2002).

A characteristic of patients carrying the R200W mutation is the absence of tumour development (Bond *et al*., 2011, Gordeuk *et al*., 2004, Sarangi et al., 2014), except for two isolated heterozygous carriers with CNS (central nervous system) haemangioblastomas (Woodward *et al*., 2007). Another case, compound heterozygous for R200W/V130L mutations, has been described with a pheochromocytoma (Capodimonti *et al*., 2012). Tumourogenesis has been driven, in this case, by the V130L mutation, the R200W being lost in the tumour. Indeed, *VHL* is a tumour suppressor gene and heterozygous carriers of *VHL* mutations have von Hippel-Lindau disease and are at high risk of multiple tumours (haemangioblastoma, pheochromocytoma, renal cell carcinoma) (Maher *et al*., 1996, Nordstrom-O'Brien *et al*., 2010, Richard *et al*., 2013). The absence of tumour

development in patients with congenital erythrocytosis carrying the R200W mutations remained unexplained. One explanation could be an insufficient dysregulation of the hypoxia pathway. The R200W mutation is indeed considered less severe than classic *VHL* mutants (Ang *et al*., 2002, Rathmell *et al*., 2004), with a function very close to the wild type protein (Couve *et al*., 2014).

– *EGLN1* gene mutations:

The first reported *EGLN1* mutation, c.950C>G, p.Pro317Arg (PHD2-P317R), was present in 3 members of the same family from 2 generations (Percy *et al*., 2006). Residue Pro317 is located in a conserved region of PHD2 that comprises the catalytic domain. It is close in primary structure to two of the three iron chelating residues at 313 and 315, and in the three dimensional structure, it is in the vicinity of the active site of PHD2. In contrast to the *VHL*-R200W mutation, which is usually associated with substantially elevated serum EPO and raised Hb, the PHD2-P317R mutation resulted in an EPO level in the normal range and subtly raised Hb. Patients with an erythrocytosis due to a mutation in the *EGLN1* gene were all found to be heterozygous. To date, eighteen different *EGLN1* mutations (in 27 patients) – comprising missense, frameshift and nonsense mutations – have been described (Gardie *et al*., 2014). The phenotypes associated with the patients carrying these mutations are fairly homogeneous and typically limited to erythrocytosis with normal to elevated EPO. However, exceptions exist; for example, there is one case with concurrent paraganglioma development (PHD2-H374R) (Ladroue *et al*., 2008), and more recently the germline mutation PHD2-A228S has also been identified in a patient with erythrocytosis and pheochromocytoma and paraganglioma (Yang *et al*., 2015). In these cases, the wild type allele of *EGLN1* has been lost in the tumour. The exact mechanism of a potential tumour suppressor role PHD2 still requires to be elucidated (Gardie *et al*., 2014).

A knockin mouse model expressing the first reported PHD2-P317R mutation recapitulates the phenotype observed in humans (erythrocytosis with inappropriately normal serum EPO levels) and demonstrates that haploinsufficiency and partial deregulation of PHD2 is sufficient to cause erythrocytosis (Arsenault *et al*., 2013).

PHD2 binds a number of proteins and the effect of the mutations on these interactions could conceivably be affected (Huo *et al*., 2012, Song *et al*., 2013, Vogel *et al*., 2010). Notably, PHD2 binds p23 that allows the recruitment of PHD2 to the HSP90 machinery to facilitate HIF-1α hydroxylation (Song *et al*., 2013). PHD2 binds FKPB38, which plays a major role in PHD2 stability (Barth *et al*., 2007). Interestingly, the PHD2 haplotype (D4E/C127S) identified in Tibetans, who are adapted to the chronic hypoxia of high altitude, diminishes the interaction of PHD2 with p23 resulting in impaired PHD2 down-regulation of the HIF pathway (Song *et al*., 2014).

– *EPAS1* gene mutations:

The first mutation in the *EPAS1* gene was described in three generations of a family with erythrocytosis (Percy *et al*., 2008). All effected individuals were heterozygous for the c.1609G>T, p.Gly537Tyr mutation in *EPAS1*. Ten heterozygous mutations have then been identified in exon 12 of the *EPAS1* gene leading to HIF-2α variants (Gale *et al*., 2008, Lorenzo *et al*., 2013, Martini *et al*., 2008, Percy *et al*., 2008, Percy *et al*., 2008, Perrotta and Della Ragione, 2008, van Wijk *et al*., 2010). These variants have been linked to idiopathic erythrocytosis and in all cases patients were diagnosed with elevated EPO levels associated with increased risk of pulmonary hypertension and thrombotic events (pulmonary embolism). Interestingly, the majority of these missense mutations (I533V, P534L, M535T, M535V, M535I, G537W, G537R, D539E and F540L) cluster close to and C-terminal to one of the two hydroxylated proline residues (Pro531) in HIF-2α. Combined biochemical and cellular assays suggest that the majority of these mutations may reduce both hydroxylation of HIF-2α by the PHDs (full-length), and subsequent recognition of (probably hydroxylated) HIF-2α by pVHL, except for mutations at Met535, which may only impair interactions with PHD2 (Furlow *et al*., 2009, Lee and Percy, 2011). This confirms that the impairment of PHD2 function is sufficient alone to cause erythrocytosis as described in erythrocytosis individuals with heterozygous *PHD2* mutations.

One patient has been described with recurrent pheochromocytoma and paraganglioma associated with a germline *EPAS1* mutation: c.1121T>A, p.Phe374Tyr (F374Y) (Lorenzo *et al*., 2013). The mechanisms of tumour occurrence with this particular mutation still need to be determined.

In conclusion, erythrocytosis may be the result of subtle dyregulation of the hypoxia pathway (weak VHL loss of function, PHD2 haploinsufficiency, perturbation but not loss of HIF-2α proline hydroxylation). The over-production of EPO seems to play a major role in the developement of erythrocytosis, however, a direct effect of the hypoxia pathway on erythroid progenitors is probable and needs to be further investigated.

In addition, because HIF plays a major role in tumour development and because some patients with mutations in the hypoxia pathway developed tumours in addition with erythrocytosis, all patients should benefit from stringent follow-up to monitor for potential tumour occurrence.

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Chapter 3
Clinical presentation

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3.1 Symptoms and Signs

The most common way that an erythrocytosis presents is as an incidental finding. The patient is being investigated or screened for some other issue and they are found to have a Hb (Hb) or haematocrit (Hct) outside the normal range prompting repeat screening and further investigation. The patient is frequently young either a child or a young adult given that these are congenital disorders and in contrast to patients with classic myeloproliferative neoplasms who typically present at much older ages.

There are a variety of somewhat vague symptoms and signs which may bring an individual to medical attention and lead to detection of erythrocytosis. Associated symptoms are feelings of

fuzziness or dizziness and awareness of increased redness of the face. Signs include a plethoric appearance, red suffused eyes and redness of the hands.

3.1.1 Family History

A family history of erythrocytosis would be expected for these congenital defects. A positive family history was present in the description of many of the original cases and in fact was a driver for further investigation, However, many cases do not have a family history of clear erythrocytosis of the history is very vague with family members in early generations having vague labels associated with polycythaemia vera. Therefore, presence of a family history in highly significant but absence of a family history does not rule out a congenital erythrocytosis.

3.1.2 Thromboses

An increased Hct leads to an increased viscosity and intuitively would be predicted to lead to thromboembolic events. Events have been described in patients with congenital erythrocytosis either as the presenting event which leads to the diagnosis or during the course of the disease (McMullin, 2010). Some of these events are severe and in unusual sites (Perrotta *et al*, 2008, Percy *et al*, 2008) in patients presenting at young ages. However, because of the rare nature of these disorders there is no clear picture of the frequency or nature of thrombotic events in congenital erythrocytosis

3.1.3 Malignancy

Lesions in the *VHL* gene was the first defect in the oxygen sensing pathway associated with congenital erythrocytosis. *VHL* gene lesions had already been described as part of the hereditary

cancer syndrome von-Hippel-Lindau disease however, no increase in malignancy has been associated with the *VHL* mutations in congenital erythrocytosis. However, several tissue specific cancers (pheochromocytoma, paraganglioma) have been documented in patients with other oxygen sensing pathway mutations: EGLN1-H374R and A228S (Ladroue, *et al*., 2008; Yang *et al*., 2015), EPAS1-F374Y (Lorenzo *et al*., 2013) and EGNL2-S61R (Yang *et al*., 2015) (see also Chapter II, Hypoxia Pathway). Examination of tumour material from patients with heterozygous EGLN1 mutation and associated paraganglioma showed loss of the wild type allele in the tumour suggesting that PHD2 may be a potential tumour suppressor gene (Ladroue *et al*., 2008; Yang *et al*., 2015).

3.1.4 Pulmonary Hypertension

Pulmonary hypertension is present in a significant percentage of patients with congenital erythrocytosis due to mutations affecting the HIF-related oxygen sensing pathway. Initially, this finding was surprising since experimental data indicated a protective effect of high serum erythropoietin with regard to the development of pulmonary hypertension under both normoxic and hypoxic conditions in mice (Weissmann *et al*, 2005). However, in a first study including patients with Chuvash polycythemia, 36 percent of patients had an increased pulmonary arterial pressure above 35 mmHg as assessed by Doppler echocardiography (Bushuev *et al*, 2006). At least one factor contributing to pulmonary hypertension in this patient group is an increased endothelin-1 serum concentration. Endothelin-1 is up-regulated by HIF2α which is overexpressed on the protein level in patients with Chuvash polycythemia. These clinical data are in agreement with results in a murine model of Chuvash polycythaemia which also showed the development of pulmonary arterial hypertension and suggested its association with HIF2α overexpression (Hickey *et al*, 2010). A subsequent clinical study including 120 pediatric and adult patients with Chuvash polycythemia confirmed these finding (Sable *et al*, 2012). In addition, it was shown that patients with previous regular phlebotomy treatment causing low serum ferritin levels had a significantly higher tricuspid regurgitation velocity indicating pulmonary arterial hypertension than those without frequent phlebotomies.

The up-regulation of HIF2α-regulated factors like endothelin-1 is obviously also underlying pulmonary hypertension in individuals with congenital erythrocytosis due to *EPAS1* (HIF2α) mutations. Two of such patients were reported in one of the first publications on congenital erythrocytosis due to *EPAS1* (HIF2α) mutations (Gale *et al*, 2008). Other, so far unpublished cases are known, some of them requiring medical treatment. Again, a murine model for HIF2α-related erythrocytosis confirmed those clinical findings associated with an increased expression of endothelin-1 (*ET1*) and other potentially involved genes like *PDGFB* and *SDF1* (Tan *et al*, 2013).

3.1.5 VHL studies

The only sizeable cohort of patients with congenital erythrocytosis, are those who were first described in the Chuvash region of Russia. This group were studied clinically and with extensive laboratory investigation and compared to control groups in the same population. VHL homozygote Chuvash patients had a more thrombosis and reduced life expectancy compared to the controls. They did not have any increase in malignancy. Blood pressures were lower in the Chuvash patients and they had increased venous varicosity (Gordeuk *et al*, 2004).

3.1.6 Physiological studies

A number of studies have been carried out looking at the physiology of the patients with oxygen sensing defects. Subjects and controls were subjected to hypoxia. Those with *VHL* mutations did not tolerate hypoxia well. They had increased pulmonary artery pressure, increased ventilation, and increased heart rate compared to controls, even under mild hypoxia but strongly accentuated under severe hypoxia (Smith *et al*, 2006). The differences are less pronounced in other oxygen sensing pathway mutations (e.g. HIF2α) but with these defects there is a different physiology compared to normal (Formenti *et al*, 2011). In addition, it was shown that phlebotomy in a patient

with HIF2α-related erythrocytosis does not lead to an improvement of the physiologic reaction to hypoxia.

3.1.7 Summary: clinical issues

An erythrocytosis may be an incidental finding, however, the typical patient with congenital erythrocytosis presents at a young age and may have a positive family history. Nonspecific symptoms and signs may be associated. Thromboembolic events and pulmonary hypertension are associated clinical events. Very tissue specific tumours may be associated with some mutations. These patients have abnormal cardiopulmonary physiology.

3.2 Treatment

3.2.1 Who should be treated

Erythrocytosis is often and incidental finding. Various forms of congenital erythrocytosis result from a genetic lesion which leads to increased EPO levels and an increased Hct. These increased levels are part of the physiological adaption to the lesion and may be required to deliver sufficient oxygen to the tissues for functioning. However, there are increased thromboembolic events associated with the increased viscosity resulting from the increased Hct and therefore consideration of reduction of the Hct and therefore the viscosity may be of therapeutic benefit. Some patients also have symptoms associated with the increased Hct which may improve with reduction of the Hct. There is no systematic evidence as to the benefit of treatments in these very rare patients.

A decision as to who to treat therefore needs to be considered in the context of the specific congenital erythrocytosis patient, their particular lesion and the associated history of events with that lesion, the patient's symptomatology, and the previous history of events in the patient.

3.3 Treatment Modalities

3.3.1 Low dose aspirin

Low dose aspirin has been shown to reduce the incidence of thromboembolic events in a randomised controlled trial in polycythaemia vera (Landolfi *et al*, 2004). This is of course an acquired disorder. However, if seems reasonable to assume that low dose aspirin may also be useful in reducing the incidence of thromboembolic events in congenital erythrocytosis where there is a similar increased Hct. Therefore it is recommended that patient with congenital erythrocytosis are treated with low dose aspirin provided that do not have a contraindication to aspirin therapy.

3.3.2 Venesection and target

The Hct can be reduced by venesection. This has been shown to be beneficial in the acquired disorder polycythaemia vera with a target Hct of 0.45 (Marchioli *et al*, 2013). In congenital erythrocytosis there is no evidence that reduction of the Hct is beneficial in reducing the incidence of thromboembolic events even though it may seem logical. Some increase in Hct may also be necessary for physiological functioning in theses patient. From a practical point of view it is impossible to reduce the Hct to 45% in patients with very extremely elevated Hcts.

However, some patients do seem to get improvement of their symptomatology with reduction of the Hct. It is therefore suggested that in patients with very elevated Hcts, with symptoms which may be associated with the Hct, or patients who have already experienced thromboembolic events, judicious reduction of the Hct by Venesection should be considered. The target for venesection is not clear but from a practical point of view the lowest target level which would be considered is 50%.

3.3.3 Pulmonary Hypertension

Patients with known mutations in genes associated with the HIF-related hypoxia sensing pathway should regularly undergo cardiologic examinations for the presence of pulmonary hypertension. Phlebotomy treatment is those patients should be considered with caution since – as shown in patients with Chuvash polycythemia – it can worsen pulmonary hypertension. In addition, one has to keep in mind that in a large observational study in Chuvash polycythemia patients the prevalence of thrombotic complications was not different among phlebotomized and non-phlebotomized patients (Gordeuk *et al*, 2004).

Due to the low total number of patients systematic data on treatment of pulmonary hypertension in these patients are lacking. In general, treatment should follow guidelines for the treatment of idiopathic pulmonary hypertension including the consideration of response testing during right heart catheterization. At least in theory, treatment with endothelin-1-receptor antagonist like bosentan or macicentan should optimal with regard to the finding of increased endothelin-1 expression in these patients.

In addition to these patients with known defects affecting the VHL-HIF-pathway, patients with idiopathic erythrocytosis should be examined as well since it is possible that some of them might have a so far unexplored defect finally also resulting in an increased HIF2α protein expression. To date, it seems as if pulmonary hypertension in the context of presumable congenital erythrocytosis might be a pathopnomonic feature.

3.3.4 High Oxygen Affinity Haemoglobins

Review of the evidence for management in high affinity Hbs suggests that in those with symptoms which could be attributed to the increased Hct, venesection should be considered. It should also be considered for those with a high oxygen affinity Hb who have had one or more previous thrombotic episodes or in asymptomatic individuals with the lesion in which another family member with the same lesion has had thrombotic problems. The recommended target Hct is less than

60% unless the symptoms or thrombosis developed at a lower Hct in which case a target of 52% is considered (McMullin *et al*, 2005).

3.3.5 Follow up

These patients need long term follow up by a haematologist in order to continue to review the need for treatment and to record any events and outcomes. It would be helpful it data and outcomes on such patients could be entered into international databases so that more information on long term outcomes is amassed.

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Chapter 4
Molecular diagnosis – protocols

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Definite diagnosis of congenital erythrocytosis can be usually made only after identification of a causative mutation by sequencing analysis of genes involved in pathology of erythrocytosis.

When performing the molecular diagnostic of a patient with erythrocytosis it is crucial starting to exclude an acquired secondary (pulmonary, renal and cardiac) or an acquired primary (PV due to *JAK2* mutations) etiology, especially if no family history of CE is reported.

The family history and the quantification of serum EPO are very useful to define the best diagnostic strategy regarding molecular studies. If available, determination of P50 can be helpful to suspect the presence of an Hb variant with high O_2 affinity.

Sequencing of the candidate genes is mandatory for a definitive diagnosis; information on the

European laboratories performing molecular studies on CE genes is available at www.erythrocytosis.org.

MPN&MPNr-EuroNet

A diagnostic algorithm to lead the diagnosis of CE was proposed by Mary Frances McMullin (McMullin *et al*., 2005) and, recently, another one for children and adolescents has been published by members of the MPN&MPNr-Euronet (Cario *et al*., 2014), based mainly in the serum EPO level and P50 determination.

A new version of the algorithm, aiming to contribute to a more efficient molecular diagnosis of CE, is proposed (Fig. I.11). This algorithm is mainly based on the assumption that:

PV is the principal cause of erythrocytosis and it should be the first etiology to be investigated when any typical PV signals (splenomegaly, thrombocytosis, leukocytosis) are present, especially if recently detected. The patient clinical history and the presence of the JAK2 V617F mutation confirm the diagnosis. If necessary, a bone marrow biopsy should be performed.

Carriers of JAK2 exon12 mutations are associated with mild PV phenotypes and could be incorrectly classified as IE;

Hb variants with high O_2 affinity are one of the commonest causes of secondary CE;

Although ECYT2 is considered a recessive disease, there are case reports of CE patients heterozygous for VHL mutations;

Although ECYT 3 and ECYT 4 are considered dominant diseases, they have been found in patients without a familial history;

Patients with ECYT type 1 presenting with EPO levels within the normal range have been reported;

This algorithm is usefully just until new sequencing methodologies are available, which allow the sequencing of all the candidate genes at the same time.

MPN&MPNr-EuroNet

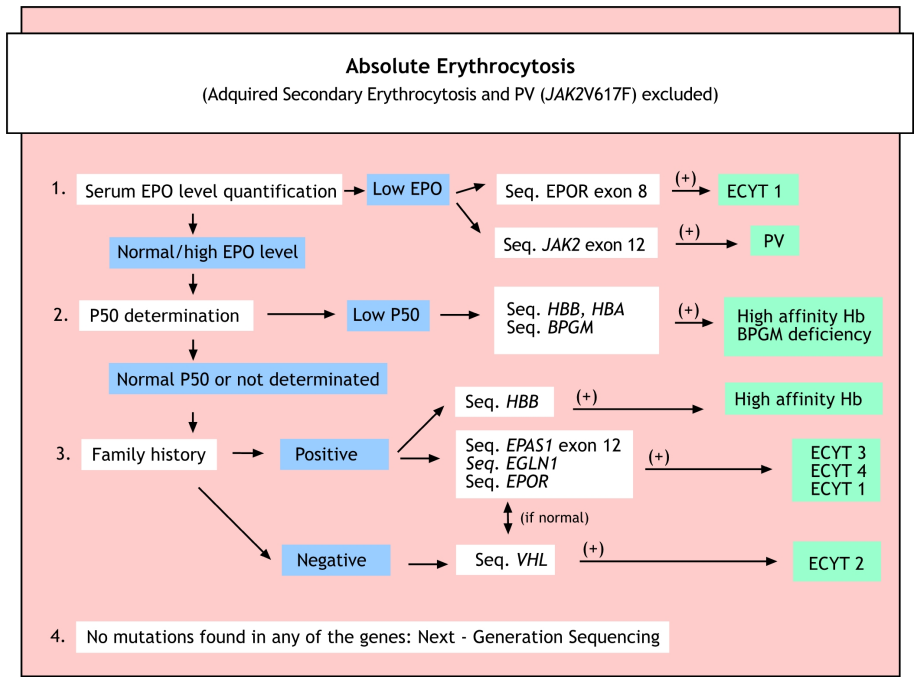

Figure I.11 Proposed algorithm to the study of absolute erythrocytosis. (+) indicates the presence of a causative mutation; Seq. means sequencing; Colors code: Blue boxes represent patient's data; white boxes represent what to do; green boxes represent the diagnosis.

This chapter will describe in detail regions of genes commonly sequenced in cases of suspected congenital erythrocytosis. In general, whole blood patient's gDNA is used for PCR and then sequencing of the products.

Due to different suppliers of PCR and sequencing reagents, the conditions described should be only considered for reference only. It should be noted, though, that most of these PCR reactions were amplified using a hot-start polymerase and sequenced using BigDye Terminator v1.1 kit (Life Technologies), which enables reads start approximately 30-40 bp from the amplicon start and up to 500-600 bp overall read length. Special requirements for several specific amplicons will be also noted.

4.1 Sequencing of genes affecting oxygen affinity

Increased oxygen affinity and sequential tissue hypoxia and secondary erythrocytosis can be, as far as it's known, caused either by variants in both beta and alpha globin chain, and also by mutations in 2,3-bisphosphoglycerate mutase gene *BPGM*. The later are though very rare. Decreased P50 value can be indicative of this type of congenital lesion.

HPLC or Hb-electrophoresis results may point to a presence of Hb variant, but many variants may remain hidden. There are more than 90 different high-affinity Hbs currently in the HbVar database, with mutations appearing in all three exons of *HBB, HBA1* and *HBA2*.

HBB primers

Primer name	Primer sequence	PCR & conditions
HBB 1 F	5'- GAGCCAAGGACAGGTACGG - 3'	Product: 461 bp Tm: 54 °C
HBB 1 R	5' - CAAAGGACTCAAAGAACCTC - 3'	
HBB 2 F	5'- AGACTCTTGGGTTTCTGA - 3'	Product: 401 bp Tm: 54 °C
HBB 2 R	5' - CATTCGTCTGTTTCCCATTCTA - 3'	
HBB 3 F	5'- CAATGTATCATGCCTCTTTACC - 3'	Product: 666 bp Tm: 54 °C
HBB 3 R	5' - GCAGCCTCACCTTCTTTCATGG - 3'	

Due to very high sequence similarity between *HBA1* and *HBA2* genes, each of them is amplified separately as a long product in non-homologous region and the same sequencing primers are used for each exon in both genes.

HBA primers

Primer name	Primer sequence	PCR & conditions
HBA1/ 2 F (*)	5'- CGCGCCCCAAGCATAAAC - 3'	Product: 809 bp Tm: 60 °C
HBA1 R	5'- CCCAAGGGGCAAGAAGCAT - 3'	
HBA1/2 F (*)	5'- CGCGCCCCAAGCATAAAC - 3'	Product: 915 bp Tm: 60 °C
HBA 2 R	5'- GGCACATTCCGGGATAGAGAG - 3'	
HBA1/2 1S (rev)	5'- CAGAGTGAGGGGTGGGGTTT - 3'	Sequencing primer
HBA1/2 2S (fwd)	5'- CCACCCTCAACCGTCCT - 3'	
HBA1/2 3S (fwd)	5'- CAGAGGATCACGCGGGTTG - 3'	

(*) common PCR primer for both HBA1 and HBA2

BPGM gene has 3 exons, with coding sequence spanning over exons 2 and 3. Only 3 mutations have been identified to date, in the coding sequence. Primers below can be used to amplify all exons (Hoyer *et al*., 2004).

BPGM primers

Primer name	Primer sequence	PCR & conditions
BPGM 1F	5'- GCCAACTCCTTACTGGTTCA - 3'	Product: 349 bp Tm: 58 °C
BPGM 1R	5' - AATGTAAACGTTCGCAACAT - 3'	
BPGM 2F	5'- CAGTTGAATATAACTTAGAC - 3'	Product: 735 bp Tm: 48 °C (*)
BPGM 2R	5' - AACCTCTAATAAGTGGTATA - 3'	
BPGM 3F	5'- TGATGTAGCACTTGCTGTG - 3'	Product: 762 bp Tm: 58 °C
BPGM 3R	5' - GTGAACTACTGATTAGAATAGTG - 3'	

(*) PCR amplification of difficult template, recommended addition of betaine 1M or 5-10% DMSO

4.2 Sequencing of genes involved in hypoxia sensing

Generally patients with normal P50 and high or inadequately normal EPO level are likely to have mutation in one of the genes of hypoxia sensing pathway. To date, mutations in three of these genes were described.

VHL gene was the first associated with hereditary erythrocytosis, comprises of three exons and homozygous or compound heterozygous mutations were found in all three of them, with p.R200W mutation being the most common. Less common heterozygous mutations were also published.

VHL primers

Primer name	Primer sequence	PCR & conditions
VHL 1F	5'- AGCGCGTTCCATCCTCTAC - 3'	Product: 528 bp Tm: 52 °C (*)
VHL 1R	5'- GCTTCAGACCGTGCTATCGT - 3'	
VHL 2F	5'- GAGGTTTCACCACGTTAGCC - 3'	Product: 486 bp Tm: 52 °C (*)
VHL 2R	5'- AGCCCAAAGTGCTTTTGAGA - 3'	
VHL 3F	5'- CAGAGGCATGAACACCATGA - 3'	Product: 462 bp Tm: 52 °C (*)
VHL 3R	5'- AAGGAAGGAACCAGTCCTGT - 3'	

(*) PCR amplification with 10% DMSO (other DMSO concentrations in ranges between 5-10% can be tested, or betaine 0.8-1.6M, Tm should be changed accordingly).
Primers described by Cario *et al.*, 2005

ENLN1 encodes prolyl hydroxylase PHD2, one of the enzimes that hydroxylate HIF and the one that has been found mutated in congenital erythrocytoses. Heterozygous loss-of-function mutations were to date found in all 5 exons, majority of them being amino acid substitutions.

EGLN1 encodes prolyl hydroxylase PHD2, one of the enzymes that hydroxylate HIF and the one that has been found mutated in congenital erythrocytoses. Heterozygous loss of function mutations were to date found in all 5 exons, majority of them being amino acid substitutions.

EGLN1 (PHD2) primers

Primer name	Primer sequence	PCR & conditions
PHD2 1aF	5'- CAGTAACGGCCCCTATCTCTC - 3'	Product: 714 bp Tm: 55 °C (*)
PHD2 1aR	5'- TGCACGGCACGATGTACT - 3'	
PHD2 1bF	5'- CATCGCTGTTCCAGGAGAA - 3'	Product: 529 bp Tm: 55 °C
PHD2 1bR	5'- GGAATGCTGCTTCTCAGCCTA - 3'	
PHD2 2F	5'- TGAAGCAGAATTCACCAGTCC - 3'	Product: 479 bp Tm: 55 °C
PHD2 2R	5'- TTTCAGTCTCAGGTATTAGGAGTGG - 3'	
PHD2 3F	5'- TTGTCCTTGCATCAGTGCAT - 3'	Product: 410 bp Tm: 55 °C
PHD2 3R	5' - GGCAGGAAAATACTCATTAGAAAGC - 3'	
PHD2 4F	5'- AGTCTCCCCTGGTTACTGTATAAAT - 3'	Product: 296 bp Tm: 55 °C
PHD2 4R	5'- AAAGCATCACCTGATTGCAG - 3'	
PHD2 SF	5'- GGAATGCAGTAGCAGAAGCTC - 3'	Product: 245 bp Tm: 55 °C
PHD2 SR	5'- AAAATGCGAACTGGTTGTCT - 3'	

(*) PCR amplification with 5% DMSO (other DMSO concentrations in ranges between 5-10% or betaine 0.8-1.6M can be tested, Tms should be then changed accordingly).

Of the three known genes encoding hypoxia inducible factor subunits, only *EPAS1* gene (or HIF2A) was found to be implicated in congenital erythrocytoses. Mutations cluster in exon 12,

near one of the sites of prolyl hydroxylation, with Gly537 being the most common codon affected. Recently, mutation near the second hydroxylation site in exon 9 was described in patient with idiopathic erythrocytosis who later developed recurrent paraganglioma, primers for both exons are included.

EPAS1 (HIF2A) primers

Primer name	Primer sequence	PCR & conditions
HIF2A 9F	5'- CCATGCATCTAGGGGAGCAGA - 3'	Product: 343 bp Tm: 60 °C
HIF2A 9R	5'- AACTCTTCCCAGCCCCAACG - 3'	
HIF2A 12F	5'- TCTGCAGGAGCTGAGTTG - 3'	Product: 631 bp Tm: 60 °C (*)
HIF2A 12R	5'- CTTACTAGTGGGTGCCTCT - 3'	

(*) PCR amplification of difficult template, recomended addition of betaine 1M or 5-10% DMSO.

4.3 Sequencing of genes of erythropoietin signalling pathway

Congenital erythrocytoses with low EPO level have currently only one validated cause, heterozygous truncations of erythropoietin receptor gene *EPOR* within exon 8, either frameshift or stop mutations. Non-synonymous substitutions at that region were also published, but clear association with CE was not yet established.

EPOR primers

Primer name	Primer sequence	PCR & conditions
EPOR 8aF	5'- CAAGTGTATCTGTGCCTCTA - 3'	Product: 641 bp Tm: 60 °C
EPOR 8aF	5'- TACTCAAAGCTGGCAGCAGA - 3'	
EPOR 8bF	5'- CTCCTGCTCATCTGCTTTGG - 3'	Product: 411 bp Tm: 60 °C
EPOR 8bR	5'- CTGAGAGAGGCCTCGCCAT - 3'	

Primers 8a also co-amplify exon 7.

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Chapter 5
Clinical cases
Presentation, diagnosis, treatment and follow-up

Coordination:
Mary Frances McMullin, Holger Cario, Tabita Magalhães Maia

Case 1 – High affinity Hb

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Clinical Presentation	A 62 years old man was hospitalized for a cerebrovascular accident.
Patient and family History	He had a history of idiopathic erythrocytosis which was known for 30 years. He was a heavy smoker with an 80 pack-year history. A daughter had two spontaneous abortions leading to discovery of a raised Hb whihc was identified as Hb Malmö.
Examination Findings	No abnormality

Laboratory parameters	Hb (g/L)	Hct (%)	RBC (x10^{12}/L)	MCV (fL)	WBC (x 10^9/L)	Pts (x 10^9/L)	Bone marrow	EPO (mU/ml)	P50 mm Hg
	199	59.9	6.26	95.8	7.7	118	Not done	23	13.8

Molecular diagnosis	*JAK2*V617F negative; *JAK2* exon12 negative. *HBB*: Carrier of Hb Malmö (beta 98 His-Gln)
Clinical follow-up and events	No complication noted. Treatment using regular phlebotomies to maintain a Hct <48% and aspirin 250mg/day.

Case 2 – Chuvash polycythemia

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Clinical presentation	A 9-year old girl came to Sweden with her family as a refugee from the Middle East in the year 2000. Except for a few episodes with sore throat, fever and vomiting she had been a healthy and normally developing child. Soon after arrival she was examined twice because of intermittent abdominal pain. Routine laboratory tests revealed a very high Hb 220 g/L and Hct 65%.
Patient and family history	At first presentation there was no known heredity for erythrocytosis. The parents and a younger brother were healthy. However, among three more siblings born after 2000, two were later found to be affected by erythrocytosis. Marriages among relatives are common in their kindred and the parents are first cousins. A first cousin of the father died of stroke at age 47 years, and two of his three children were born with neurological and mental defects (details of these cases are unknown). Our patients' paternal grandfather at age 72 years suffered a myocardial infarction and passed away due to stroke. Several other relatives are reported to be healthy but have not been investigated for possible erythrocytosis.
Examination Findings	A normally lively girl without signs of severe illness. No splenomegaly or hepatomegaly, but on abdominal palpation, diffuse tenderness was found. The paediatric haematologist noted conjunctival vascular congestion. Computer tomography of the thorax and abdomen were performed without abnormal findings. Echocardiography showed normal cardiac function and no signs of pulmonary hypertension. Repeated electrocardiograms have shown right axis deviation. Blood pressure at a visit to the hospital lately was 110/60.
Laboratory parameters	At presentation, Hb was 220 g/L and Hct 65%. White blood cell and platelet counts were normal, as was the differential count. EPO was normal when initially analyzed, but elevated to 33 IU/L (ref 2.6-18.5) lately in the presence of iron deficiency. Hb fractionation showed a mildly elevated HbF but normal HbA2. A bone marrow biopsy showed enhanced erythropoiesis but was otherwise normal. LAP-score 38 (ref 20-110). *JAK2* V617F mutation status negative. PK-INR, APTT, Factor 8, von Willebrand factor, fibrinogen and platelet aggregation tests normal. P-PAI-1 elevated 20 kIE/L (ref <15). D-dimer elevated 1.0 mg/L (ref <0.5). Homocysteine 14.3 mmol/L ("level for intervention" >15).

Molecular diagnosis	Electrofocusing gave a normal result and mass spectrometry showed no evidence for a Hb variant. Sequencing of the *EPO-receptor* gene showed no mutation in cd 355-490. In 2012, sequencing of exon 2 and 3 of the *VHL* gene showed a homozygous C to T transition in exon 3, corresponding to the amino acid change Arg200Trp. Also all other family members showed this so called Chuvash polycythemia mutation: our index patient and two younger siblings are homozygous, whereas the parents, two other siblings and the only investigated cousin are heterozygous.
Clinical follow-up and events	In addition to the investigations for erythrocytosis, the child was also followed for chronic urticaria and suspected food allergy. The allergic problems were treated with antihistamines and diminished over the years. From 2003 to 2011 she was treated by repeated phlebotomies or sometimes erythrapheresis, totally 31 times during 8 years, with an aim to keep the Hb below 165 g/L. She and her parents have reported that she often felt very weak and sometimes fainted immediately after these treatments. Our patient has no symptoms suggestive of pulmonary hypertension such as abnormal strain when physically active, and the oxygen saturation is normal. At 13 years of age she experienced relatively sudden hearing loss. Audiological examination showed bilateral sensorineural hearing impairment, most severe on the left side. Magnetic resonance imaging showed no abnormalities. With a hearing device she has been able to complete school. She was hospitalized for two days due to vertigo and tinnitus in 2011. A younger brother who is heterozygous for the *VHL* mutation also has hearing impairment of the same kind, with onset at 9 years of age. No apparent cause for the hearing loss was found. Further audiological evaluation of Chuvash-mutated patients is warranted in order to see if hearing loss is overrepresented in this population. There have been no overt thrombotic events in our patient or her parents and siblings. At 13 years of age our patient had a non-severe bleeding suspected to be due to an anal fissure, treated with local ointment. At 20 years of age she was examined by a surgeon for haemorrhoids which were present around the whole circumference. Notably the (heterozygous) father had problems with haemorrhoids from age 35 years and was operated for these at age 45 years. The paternal grandfather was operated for lower extremity varicose veins at age 40 years. Varicose veins were earlier reported to be overrepresented in homozygous Chuvash polycythaemia patients in the Republic of Chuvashia and associated to high serum VEGF levels.

In our patient also a moderate general bleeding tendency with mmenorrhagia, easy bruising, bleeding from wounds and gums and prolonged bleeding after a tooth extraction has been noted. A mild bleeding tendency with easy bruising is present in the two other homozygous siblings. The younger homozygous sister also has menorrhagia. The heterozygous sister has visited a doctor for repeated nose bleeds since childhood, but has no other bleeding tendency. Presently it is not known if the bleeding tendency is due to the *VHL* mutation or other maybe inherited factors / deficiencies.

After the diagnosis Chuvash polycythaemia was made in 2012 in our patient, no therapeutic bloodletting has been performed in spite of high haematocrit values. Such treatment would however be considered if symptoms such as headache or fatigue would be substantial. At present, iron deficiency probably due to menorrhagia keeps the Hb below 180 g/L and EVF below 55% in our index patient. Genetic counselling has been offered, and we strive to characterize the haemostasis situation better in view of prospects of future pregnancy.

Case 3 – *VHL* homozygous (homozygous K196E)

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Clinical presentation

We present an 82 year-old Spanish female with elevated Hb observed first at 33 years of age.
She has Type 1 diabetes mellitus, polyarthritis.
As she gave a history of headaches and she has been managed by regular phlebotomy treatment since presentation.
She had no history or evidence of thrombotic complications or cancer.

Patient and family history

Her parents are second-degree relatives.
She had a doubtful family history of polycythaemia and evaluation of her two asymptomatic children, revealed normal Hb values.

Examination findings

Her spleen had a normal size on ultrasound.

Laboratory parameters 196

Hb (g/L)	Hct (%)	RBC (x10^{12}/L)	MCV (fL)	WBC (× 10^9/L)	Pts (× 10^9/L)	EPO (mU /ml)	P50 (mm Hg)
	60	7.0	91.3	6.6	221	27.30	Normal

Electrophoretic techniques and cation exchange HPLC did not revealed any abnormal Hb.
Culture assays in methylcellulose. Evaluation of erythroid progenitors after responses to EPO: IL3+ GM-CSF

With EPO	Without EPO
BFU-E : 81/10^5CMN	BFU-E : 0/10^5CMN
CFU-GM 10/10^5CM	CFU-GM 21/10^5CMN

Molecular diagnosis

Sequencing of the *VHL* gene revealed a new homozygous missense mutation (*VHL* c.586A>G) leading to a change of a Lysine into a Glutamic acid at CD196 (p.Lys196Glu; K196E).
Her children were heterozygous for this mutation.

Clinical follow-up and events

The patient remains asymptomatic and her Hb stabilized at around 164g/L since the age of 60 years associated with iron deficiency.

Case 4 – *VHL* homozygous P138L

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Clinical presentation	A 15-year-old girl of Asian Indian extraction of Punjabi ethnicity with high Hb (180-200 g/L), slightly elevated EPO and no evidence of high affinity Hb or 2,3-DPG deficiency as determined by a normal p50, and absence of *JAK2*^{V617F} or exon 12 *JAK2* mutations.
Patient and family history	She is a non smoker, nor is she exposed to second hand smoke. There are no known drug allergies. There are no current medications. Parents are hematologically normal, the same ethnicity but not known to be related and 19-year old brother is normal. No VHL syndrome malignant or benign tumors have been encountered in the propositus' parents or in their extended family.
Examination findings	Physical examination reveals no splenomegaly, and normal skin color and no other abnormalities. The propositus has had no relevant symptoms.

Laboratory parameters

08/2013; 03/2013; 09/2012; 05/2012

Hb (g/L)	Hct (%)	RBC (x10^{12}/L)	MCV (fL)	WBC (× 10^9/L)	Pts (× 10^9/L)	EPO (mU /ml)	P50 (mm Hg)
194	58.1	6.61	87.9	10.5	226	N/A	N/A
192	57.8	6.58	87.9	8.4	237	N/A	N/A
193	59.2	6.71	88.1	10.5	215	N/A	N/A
188	57.4	6.53	87.9	9.4	251	40	normal

Molecular diagnosis	Patient is homozygous for a c.413 C > T mutation, which exchanges Pro for Leu at amino acid 138 (p.Pro138Leu; P138L). Her parents are *VHL*^{P138L} heterozygous. The binding of mutated pVHL to the HIF1α peptide showed decreased affinity to HIF1α peptide, with only 26.6% of the VHL protein bound. By comparison, the wild-type VHL protein (100%) and also proteins with other homozygous polycythaemic mutations (H191D, R200W) have much stronger affinity for the HIF1α peptides, with 49.0% and 62.2% of the bound VHL protein, respectively. These data indicate that the P138L mutation specifically reduced the affinity of pVHL for HIF1α, resulting in a reduced rate of ubiquitination under normoxic conditions. Further, VHL^{P138L} protein has decreased stability *in vitro*. Similarly to what was reported in Chuvash polycythemia and some other instances of HIFs up regulation, *VHL*^{P138L} erythroid progenitors are hypersensitive to erythropoietin (Figure 1).
Clinical follow-up and events	The patient has been compliant with low dose aspirin on a daily basis. She currently has no specific complaints. A phlebotomy program has not been initiated as the patient is asymptomatic.

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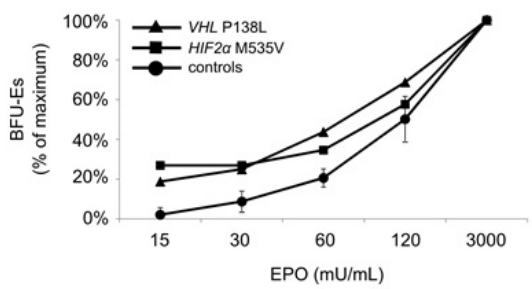

	No. of BFU-E colonies		
EPO (mU/mL)	*VHL* P138L	*HIF2α* M535V	controls
15	6	7	1 ± 1
30	8	7	2 ± 1
60	14	9	3 ± 1
120	22	15	7 ± 2
3000	32	26	14 ± 6

Figure I.12 Response of BFU-E erythroid progenitors to EPO. EPO dose-response curves derived from the homozygous VHL^{P138L} patient (▲), patient with heterozygous gain-of-function $HIF2\alpha^{M535V}$ mutation (■) and a healthy controls (●; n = 8, T bars = SD). VHL^{P138L} affected erythroid progenitors display hypersensitivity to low concentration of EPO (15 - 60 mU/mL). There was relatively higher number of BFU-Es in comparison with healthy controls (number of colonies ± SD) in all analyzed EPO concentrations. The assays of VHL^{P138L} and $HIF2\alpha^{M535V}$ erythroid progenitors were not done concomitantly.

Case 5 – *VHL* Homozygous VHL D126N

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Steven and Alexandra Cohen Children's Medical Center, New Hyde Park, NY.

Clinical presentation

An 8 month old male of Bangladeshi ethnicity, presented in the paediatric haematology clinic for work up of polycythaemia (haematocrit 67.9%) picked up by a paediatrician on routine blood work. The EPO level of the patient was dramatically elevated at 2407 mIU/ml (normal <32 mIU/ml) and imaging of the abdomen revealed multiple hepatic hemangiomas.

Patient and family history

His parents (first cousins) were both healthy, in their early twenties and neither them nor their extended family had any *VHL* associated tumours or polycythaemia.

Examination findings

The patient had failure to thrive (both weight and height <5th percentile) on presentation. Pertinent positive findings were presence of auscultatory bruit over anterior fontanelle and bounding pulses.

Laboratory parameters

Hb (g/L)	Hct (%)	RBC (x10^{12}/L)	MCV (fL)	WBC (× 10^9/L)	Pts (× 10^9/L)	EPO (mU /ml)	P50 (mm Hg)
191	67.9	7.52	90.3	7.3	182	2407	Normal

Molecular diagnosis

Homozygous mutation in *VHL* exon 2, c.376G>A, p.Asp126Asn (*VHL*^{D126N}) has been identified by Sanger sequencing. Both parents were heterozygous for the some *VHL*^{D126N} mutation.

Clinical follow-up and events

He was started on a biweekly phlebotomy regimen to maintain a Hct <55% with iron repletion and prophylactic anticoagulation with dalteparin due to his high risk of thrombosis. He developed severe pulmonary hypertension at 16 months of life which was confirmed by cardiac catheterization. At this time he presented with respiratory distress secondary to *Parainfluenza virus* and needed ventilation and inotropic support and eventually was treated with inhaled nitric oxide. When he was 2 years old another episode of *Parainfluenza* infection led to worsening of the pulmonary hypertension and new onset seizures. He ultimately became unresponsive and died. MRI of his brain prior to his demise revealed extensive cerebral, cerebellar and brain stem infarctions.

Case 6 – Erythrocytosis in a child with heterozygous *VHL* mutation

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Clinical presentation	We describe a 12 year old male, born in 1975, who had been evaluated for epistaxis, dizziness and headache since the age of 4 years. At the time of the first clinical evaluation, sustained increased Hct (60%) and Hb (195 g/L) were found in absence of leucocyte and platelet counts alterations.
Patient and family history	The child did not have any family history of erythrocytosis. His physiological and medical history were unremarkable. There were no recent treatments.
Examination findings	The physical examination showed facial plethora and ruddy of hands and feet without palpable splenomegaly. There was no lymphadenopathy. Chest and abdominal examinations were normal as confirmed by chest radiography, ECG, echocardiogram and abdominal ultrasound. There were no prothrombotic conditions. Neither variants nor unstable Hb forms were detected. Erythrocyte osmotic fragility test and *in vitro* lysis test were normal as well as iron balance, vitamin B12 and folate levels. Mild indirect hyperbilirubinemia was present with normal transaminases.
Laboratory parameters	Bone marrow biopsy was hypercellular with markedly increased erythropoiesis and a mild focal increase of reticulin. No *BCR/ABL* rearrangement was demonstrated by RT-PCR. Spontaneous erythroid colony growth (EEC), performed on mononuclear cells from peripheral blood using standard methods, was negative. Cytogenetic analysis showed normal 46, XY karyotype. The red blood cell mass, evaluated by autologous infusion of radio-labelled red blood cells, was extremely high (57 ml/kg). While the overall features suggested a myeloproliferative neoplasm compatible with Polycythaemia Vera (PV). This diagnosis was not supported by a marked increased serum erythropoietin levels (1200 – 4000 U/L n.v. 5-20). Confirming the diagnostic clues, in 2006 the study of *JAK*2 gene failed to find V617F or exon 12 mutations.

Molecular diagnosis

Increased serum erythropoietin levels are found in congenital erythrocytosis due to alteration of homeostatic mechanisms of oxygen sensitivity, the most common being the *VHL* gene mutations; we thus studied the *VHL* gene of the proband and of his family.
The study showed the presence of heterozygous missense mutation involving the VHL amino-acid residue in position 144 (c.430G>A; p.Gly144Arg); no other *VHL* mutations were detected at the DNA level; the transcripts of both VHL wild-type and mutated allele were documented at mRNA level. The same mutation was present in the mother as well as in one of the propositus' sisters (30 years old). Molecular analysis of the *VHL* gene in the other members of the family was negative.
The VHL alteration found in our patient was never described before in VHL disease or in patients with erythrocytosis, although VHL codon 144 has been previously found to be altered by missense mutations in patients with VHL-related tumours (www.umd.be:2020/ and HGMD).
A complete clinical work-up (ophthalmological and audiological evaluation, brain, spinal and abdominal CT scan/MRI and blood and urine measurement of catecholamines) failed to identify possible undetected VHL-related manifestations. The Gly144Arg mutation does not seem to cause VHL disease in the patient and his relatives.
Interestingly, while the proband has congenital erythrocytosis, both his mother and sister carrying the same heterozygous mutation, show no signs of erythrocytosis, and hence the mutation itself does not correlate with either phenotype.
In following years, since molecular alterations other than VHL mutations have been described in congenital erythrocytosis, we searched for prolyl-hydroxylase-2 (*EGLN1/PHD2*) and hypoxia-inducible factor 1-alpha (HIF-1α) genes, but failed to find any abnormality. We looked also at the erythropoietin receptor (EPO-R), which was also normal (even if serum EPO was not consistent with the latter gene mutation).
Other unknown factors might contribute to determine the polycythaemia phenotype in the propositus. The molecular mechanism of erythrocytosis in this case remains to be elucidated.

Clinical follow-up and events

Since diagnosis, the patient underwent regular phlebotomies (7-8 per year) to maintain the haematocrit between 45 and 50% (18-20) thus inducing a progressive microcytaemia (MCV up to 62 fL). At the last follow-up, red blood cells are 8.39 x 10^{12}/L and Hb 134 g/L and no increase in white blood cells (8.3 x 10^9/L) and platelet count (253 x 10^9/L) have been documented.
Since the beginning of smoking (10-15 cigarettes per day) at the age of 14 years, mild but progressive signs of pulmonary emphysema were evident at physical examination without abnormal peripheral oxygen saturation.
Palpable splenomegaly, 2 cm below costal margin, and mild hepatomegaly were observed two years after diagnosis. A progressive increase of spleen and liver size has been documented in subsequent years. Interestingly, at the abdomen ultrasound performed in 2003, a spleen area compatible with a previously asymptomatic splenic infarction was observed.
Last physical evaluation in March 2014 documented palpable spleen 6 cm, and liver 5 cm, below costal margin and last ultrasound showed enlarged liver with rounded profiles and splenomegaly with bipolar diameter of 17 cm.
At present, the patient exhibits a congestive face and ruddy of hands and feet but has not, as well as during all follow-up, erythromelalgia or other microvascular disturbances, i.e. dizziness, headache, aquagenic pruritus. He has had no thrombotic complications other than spleen infarction nor haemorrhages.
Therefore, over 27 years of follow-up, this patient did not develop clinically significant complications from increased haematocrit and its rheological effects. He has not evolved into myelofibrosis or acute leukaemia as in polycythemia vera. Moreover, no tumour-related signs as in VHL disease have been documented.
How the heterozygous *VHL* mutation, without other structural or transcriptional alterations, can induce an erythrocytotic phenotype in our patient but not in his relatives, is still now unresolved.

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Case 7 – *VHL* compound heterozygous

MF McMullin
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Clinical presentation	The male propositus was found at the age of 9 years to have a raised Hb.
Patient and family history	His father had a splenectomy (reason unknown). A brother and sister had normal blood counts recorded
Examination findings	At presentation there were no abnormal findings on examination. An ultrasound of the abdomen revealed a duplex left kidney but was otherwise normal. At the age of 27 years she was first noted to have 2cms splenomegaly This has remained at about the same size since first noted.

Laboratory parameters

Hb (g/L)	Hct (%)	RBC (x10^{12}/L)	MCV (fL)	WBC (× 10^9/L)	Pts (× 10^9/L)	EPO (mU /ml)	*JAK*2 V617F
232	0.66	na	89	na	176	394	Neg

BFU-E. No endogenous colonies

Molecular diagnosis	*VHL* gene Compound heterozygous: C562G (Val 188Met) and C598T (Arg200Trp).
Clinical follow-up and events	He has been venesected once every 2 months as an adult to maintain Hct less than 0.47. Headaches have been reported which resolved with venesection.

Case 8 – Germline *VHL* truncated mutation in patient with erythrocytosis

Betty Gardie and Stéphane Richard
Laboratoire de Génétique Oncologique de l'Ecole Pratique des Hautes Etudes (EPHE), Villejuif, France.

Clinical presentation	The patient was first referred in 1983 (laboratory of William Vainchencker), as a 20 years old male with an haematocrit at the upper end of the normal range on routine blood examination (see table below). The presence of hypercalcemia and hyperphosphoremia was suggestive of primary hyperparathyroidism.
Patient and family history	The other family members presented neither signs of erythrocytosis nor characteristic lesions of von Hippel-Lindau disease (pedigree represented in Figure 1).
Examination findings	Clinical and laboratory examinations confirmed the diagnosis of true polycythaemia, but routine and specialized tests failed to identify a specific cause. In vitro EPO-dependent erythroid colony-forming unit (CFU-E) growth pattern ruled out the diagnosis of Polycythaemia Vera. A benign parathyroid adenoma was diagnosed and resected. In 1988 arterial hypertension was diagnosed, no urinary excretion of metanephrins was detected. In 2005, a magnetic resonance imaging (MRI) detected 7 renal lesions inferior to 1 cm. In 2012, MRI confirmed the benign character of these lesions (renal cysts). MRIs did not detect any other suspicious lesions in the central nervous system, or in adrenal glands, but revealed two small benign pancreatic cysts, that may be Intraductal Papillary Mucinous Neoplasia (IPMN) of the Pancreas.

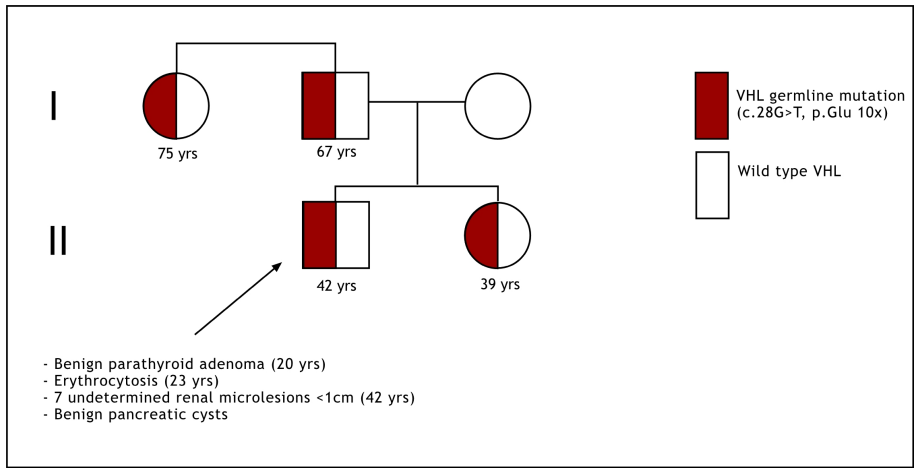

Figure I.13 Pedigree tree of the proband's family. The proposus is indicated by an arrow.

Laboratory parameters	Hb (g/L)	Hct (%)	RBC (10^{12}/L)	EPO (mU /ml)
	190	58.3	6.5	22.3 to 38

Molecular diagnosis	In 2005, the germline DNA was sequenced for genes of the hypoxia sensing pathway potentially implicated in congenital erythrocytosis with high serum EPO level (*VHL, PHD1, PHD2, PHD3, HIF2A*). The sequencing identified a unique c.28G>T, p.Glu10X heterozygous variation in the *VHL* gene (named *VHL*-E10X thereafter). Genotyping of the patient' relatives identified 3 more carriers of *VHL*-E10X mutation in the family. Stringent medical examination of the mutation carriers confirmed that only the proband developed symptoms (erythrocytosis and cysts) (Figure 1). In addition no *JAK*2V617F or mutations in the exon 12 of JAK2 could be detected. The absence of medical signs in the relatives illustrates the low penetrance of this mutation. Notion of variable penetrance is a common characteristic of autosomal dominant inherited disease (implying the influence of the environment and the importance of the genetic background with additional genetic variations). However, this is not a usual observation in von Hippel-Lindau disease, especially in case of truncated mutations that predispose to a severe disease in the majority of cases (high incidence of CNS and retinal haemangioblastomas, renal carcinoma and pheochromocytoma). To explain the low severity of the phenotype developed by the *VHL*-E10X carriers (either no symptom or erythrocytosis with benign cysts), we examined the location of the truncated mutation. Indeed, the mutation is located between the two codons of translation initiation (Figure 2). The mutated allele is therefore still able to produce the short pVHL19 isoform that contains the major domains required for HIF regulation. Then, the E10X mutation potentially induce minor defect regarding the regulation of the hypoxia pathway.
Clinical follow-up and events	The patient was regularly treated with phlebotomy to maintain the haematocrit below 45%. Hypertension was treated with inhibitors of Angiotensin Converting Enzyme (Enalapril maleate) and vasodilatator (Nicardipine chlorhydrate).

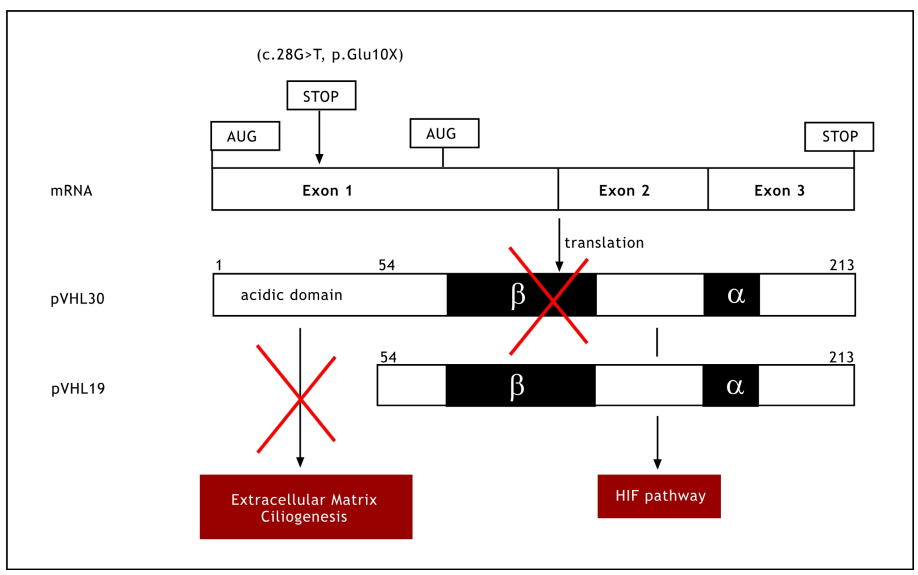

Figure I.14 Structure of the VHL mRNA and consequences of the E10X mutation on the initiation of the translation of the two protein isoforms of VHL. The suspected role of the different domains is indicated.

Case 9 – *EPOR* truncated mutation

MF McMullin
CCRCB, Queens University, Belfast, N. Ireland.

Clinical presentation

A 16 year old male presented with heat intolerance, epistaxis and headaches.

Patient and family history

There was no family history noted.

Examination findings

No abnormalities were identified on examination.
Ultrasound of the abdomen did not reveal any abnormalities.

Laboratory parameters

Hb (g/L)	Hct (%)	MCV (fL)	WBC ($\times 10^9$/L)	Pts ($\times 10^9$/L)	EPO (mU/ml)	P50 (mmHb)
205	60	92	4.1	211	<5	24.5

Red cell mass was 133% of predicted. BFU-E culture studies showed small numbers of spontaneous BFU-Es in serum-free culture. The response of BFU-E progenitors to IL-3 and IFN in the presence of erythropoietin was similar to that of polycythaemia vera.P50 24.5mmHg (normal)

Molecular diagnosis

Sequencing of exons 7 and 8 of the *EPOR* gene revealed a mutation which converts codon 439 to a stop codon: c.6002G>A, p.Trp439X.

Clinical follow-up and events

He has now been followed for 16 years since diagnosis. Venesection of one unit about once every 6 months required. No events have occurred.

Case 10 – A *de novo* mutation (c.1142_1276del35) of the *EPOR* Gene

Milen Minkov[1], Petra Zeitlhofer[2], Oscar Haas[2], Lourdes Florensa[3], Holger Cario[4]

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Clinical presentation	A 13.5 year old daughter of immigrants from Tschetschenia was referred for a second opinion for a primary sporadic congenital erythrocytosis.
Patient and family history	The family moved to Austria recently, but the parents reported that the blood counts and Hb level were normal until the age of three. Elevated Hb levels were first detected at the age of four years and since then the average Hb value was 165-170 g/L, with a documented maximal level of 190 g/L. The parents reported further on recurrent episodes of headaches and dizziness. Strenuous physical activities, including physical exercises at school, were avoided since early age due to exercise intolerance. In the country of origin the elevated Hb was assumed to result from a congenital heart defect. Otherwise the growth and development over the childhood were apparently unremarkable. The parents are not consanguinous and both are healthy.
Examination findings	Clinical examination, was unremarkable and in particular there were no signs of plethora, neurological deficits, or dyspnoea at rest. Echocardiography, ECG, chest x-ray, pulmonary function tests, sonography of the abdominal organs, all unremarkable. Exercise ECG discontinued at 50 Watt due to dizziness, dyspnoea and headache.

Laboratory parameters	Hb (g/L)	Hct (%)	RBC (x10^{12}/L)	MCV (fL)	WBC (×10^9/L)	Pts (×10^9/L)	EPO (mU/ml)	*JAK*2 V617F	*JAK*2 ex12
	182	55	6.81	81	6.1	226	<1	neg	neg

Molecular diagnosis	A novel deletion NM_000121.2: c.1242_1276del35 (p.Ser415HisfsX18) was found in exon 8 of EPOR. This deletion was not present in the parents, thus confirming a spontaneous deletion in the index case.

Clinical follow-up and events	There were no thrombotic or haemorrhagic events during a follow-up time of 4 years and no specific interventions.

Case 11 – Family with *EGLN1* (PHD2) mutation

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Laboratoire d'hématologie, CHU Dijon, France.

Clinical presentation	A 31 years old woman was referred to the hospital after the discovery of an isolated erythrocytosis without symptoms.										
Patient and family history	No personal history of note. A sister has an erythrocytosis associated with fatigue, headaches, acquagenous pruritus, "pins and needles" in the hands.										
Examination findings	No abnormality was revealed										
Laboratory parameters	Hb (g/L)	Hct (%)	RBC (x10^{12}/L)	MCV (fL)	WBC (×10^9/L)	Pts (×10^9/L)	Bone marrow	EPO (mU/ml)	*JAK2* V617F	*JAK2* exon12	P50
	174	51	5.17	98.5	7.3	219	normal	8.9	neg	neg	26.8
Molecular diagnosis	Heterozygous *EGLN1* W334R mutation										
Clinical follow-up and events	No complication has been noted. She has been treated with low dose (100 mg/day) aspirin only. No phlebotomy has been required.										

Case 12 – Family with a *EGLN1* (PHD2) mutation

MF McMullin
Institution: CCRCB, Queens University, Belfast, N. Ireland.

Clinical Presentation	The propositus presented at the age of 26 years with superficial thrombophlebitis when she was noted to have a raised Hb
Patient and family history	The father of the propositus presented with intermittant claudication aged 45 years. He was treated for erythrocytosis (Hb 180g/L) by venesection from that time. A smoker, he died of lung cancer aged 61 years. A brother presented at age of 30 years with borderline erythrocytosis (Hb 175g/L) The mother of the propositus was found to have an normal Hb.
Examination findings	On examination no abnormalities were noted

Laboratory parameters

Hb (g/L)	WBC ($\times 10^9$/L)	Pts ($\times 10^9$/L)	Bone marrow	EPO (mU /ml)	P50
180	11	268	normal	6.3	normal

Molecular diagnosis	An heterozygous mutation in EGLN1/PHD2 has been identified by sequencing: c.950C>G, p.Pro317Arg. The mutation is present in the propositus and her brother. It was also identified in tumour tissue from the father (after his death).
Clinical follow-up and events	

Case 13 – *PHD2* mutation in patient with erythrocytosis and paraganglioma

Betty Gardie and Stéphane Richard

Institution: Laboratoire de Génétique Oncologique de l'Ecole Pratique des Hautes Etudes, Villejuif, France.

Clinical Presentation	The patient was first referred in 1988, a 30 year old male with an haematocrit in the upper normal range on routine blood examination (see table below). A mild hypertension was intermittently observed with urinary excretion of metanephrins in the upper normal range.
Patient and family history	The other family members (excepted parents, deceased) did not present with either erythrocytosis or tumours (pedigree represented in Figure 1).
Examination findings	Clinical and laboratory examination confirmed the diagnosis of true polycythaemia, but routine and specialized tests failed to identify a specific cause. *In vitro* EPO-dependent erythroid colony-forming unit (CFU-E) growth pattern ruled out the diagnosis of Polycythaemia Vera. Abdominal ultrasonography repeated yearly remained normal until 2001. At that time, the patient was 43 years old, and a para-aortic mass was discovered then confirmed by computed tomography (CT) and magnetic resonance imaging (MRI). A solid 3.5 x 3.5 cm encapsulated, red-brown tumour was resected and cut section revealed two additional small nodules, 13 mm and 8 mm large. On microscopic analysis, the tumour displayed typical aspects of paraganglioma. Incidentally, mean corpuscular volume (MCV) and ferritin level remained surprisingly within the normal range during the follow-up despite the phlebotomy regimen, and haemochromatosis was suspected.

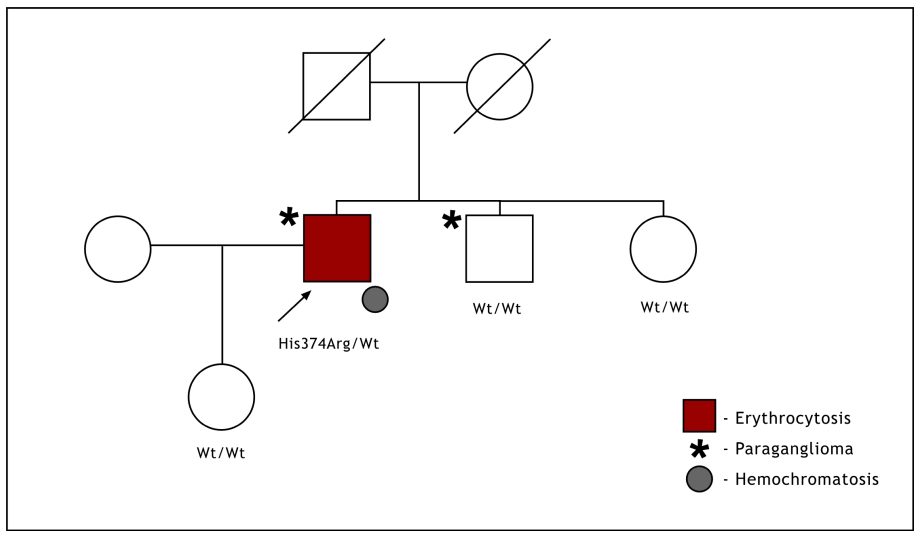


Figure I.15 Pedigree tree of the proband's family. The patient reported here is indicated by an arrow. Results of the genotyping for the *PHD2* gene are indicated. Wt: wild type allele.

Laboratory parameters	Hb (g/L)	Hct (%)	RBC (10^{12}/L)	WBC (10^9/L)	Pts (10^9/L)	Bone marrow	EPO (mU/ml)	P50 (mmHg)
	202	61.6	6.2	6.8	162	Erythroid hyperplasia, without fibrosis	18	31

Molecular diagnosis

The hypothesis of a potential VHL disease or hereditary paraganglioma was ruled out by sequencing of *VHL, SDHB, SDHC* and *SDHD* genes in germline DNA of the patient.

The germline DNA has been sequenced for genes of the hypoxia sensing pathway potentially implicated in congenital erythrocytosis with high serum EPO level (*PHD1, PHD2, PHD3, HIF2A*). The sequencing identified a unique c.1121A>G, p.His374Arg heterozygous variation in the *PHD2* gene (named H374R thereafter). None of the three first degree relatives of the patient was a carrier of the *PHD2*-H3734R mutation (Figure 1).

The patient's tumour was sequenced for the *PHD2* gene. The mutant allele was detected and the signal for the wild type allele was significantly reduced on the chromatogram. The loss of heterozygosity (LOH) was confirmed by microsatellite analysis of the *PHD2* region which showed unizygosity for all the markers tested.

Consequences of the amino-acid change were analyzed *in silico* and all bioinformatics software predicted a damaging effect. Indeed, Histidine 374 is a highly conserved residue and H374 amino acid is one of the three critical residues that coordinate binding of Fe(II) ion the catalytic domain of PHD2.

Functional consequences of the H374R mutation on PHD2 activity were evaluated using a reporter transcription assay (Ladroue *et al*., 2008 and 2012) and an *in vitro* HIF hydroxylation assay (Ladroue *et al*., 2012). The H374R substitution totally impaired the PHD2 function (loss of the PHD2 capability to induce HIF-1a hydroxylation and to inhibit HIF activity).

The *HFE* gene (involved in haemochromatosis) was also found mutated in the patient (homozygote for the C282Y mutation). The *HFE* gene was then sequenced for all the family and the brother of the proband was found homozygous for the C282Y mutation and is now treated for hemochromatosis. The *HFE* gene has no causal relationship with polycythaemia.

Clinical follow-up and events

The patient was regularly treated with phlebotomy to maintain the haematocrit below 45%.
The hypertension was treated by atenolol. After the first paraganglioma resection (in 2001), arterial pressure returned to normal values and atenolol was discontinued, whereas haematocrit stabilized within the normal range without further phlebotomy, until 2003. At that time, as urinary excretion of metanephrins was upper the normal range and haematocrit raised again, a recurrence of the tumour was suspected, finally substantiated on Iodine-131 metaiodobenzylguanidine (MIBG) scintigraphy scans in 2004. A second intervention allowed a complete resection of the tumour, which histological aspect was similar to the previous one.
From 2004 to 2008, the patient's status remained unchanged, with a slight elevation of haematocrit and blood pressure, both requiring specific treatment with phlebotomy and anti-hypertensive drugs. Repeated CT and MIBG scans did not detect tumour recurrence until now, although urinary excretion of metanephrins remains twice above normal range during this period of time.

Case 14 – Family with a *EPAS1* (HIF2A) mutation

MF McMullin
CCRCB, Queens University, Belfast, N. Ireland.

Clinical presentation	A male presented at the age of 23 years having been found to have an elevated Hb							
Patient and family history	The mother of the propositus had previously presented at the age of 35 years with an elevated Hb (163g/L) and an elevated red cell mass. At the age of 63 years she had a myocardial infarction. The maternal grandmother of the propositus had presented at the age of 54 years with an eleavted Hb (191g/L). She was venesected 2/3 times per year until the age of 81 years when her Hb fell and up to the age of 89 years she no longer needed venesected. Father and brother of the propositus have normal full blood counts.							
Examination findings	No abnormalities were noted on examination							
Laboratory parameters	Hb (g/L)	Hct (%)	MCV (fL)	WBC ($\times 10^9$/L)	Pts ($\times 10^9$/L)	EPO (mU/ml)	P50	
	217	64	92	7.2	226	31.1	normal	
Molecular diagnosis	A germline heterozygous mutation in *EPAS1/HIF2A* has been identified by sequencing: c.1609G>T, p.Gly537Trp.							
Clinical follow-up and events	The patient from 23 to 42 years of age was venesected 2/3 times a year to maintain the Hct below 55%. At 42 years of age he had a deep venous thrombosis and was subsequently venesected to maintain the Hct below 45%.							

Case 15 – HIF2α heterozygous (heterozygous *EPAS1* p.Gly537Arg)

Holger Cario
Dept. Of Pediatrics and Adolescent Medicine, University Medical Center Ulm, Germany.

Clinical presentation	A 14-year old girl, with erythrocytosis diagnosed as being independent from disturbed pulmonary function at age 7, presenting with progressing fatigue and headaches despite stable haematocrit and ASA treatment. Echocardiography revealed right heart hypertrophy with an estimated RV pressure of >45 mmHg, normal RV function (TAPSE 19mm). Pulmonary arterial hypertension (PAH) was confirmed by right heart catheterization (RV pressure without oxygen 66 mm Hg at a systemic systolic arterial blood pressure of 128 mmHg, RV pressure with 100% oxygen 51 mm Hg at a systemic systolic arterial blood pressure of 134 mmHg) which by angiography also indicated pulmonary vasculopathy.
Patient and family history	Born after 26 gestational weeks per section due to intra-uterine growth retardation caused by placental thrombosis due to suspected antiphospholipd antibody syndrome of the mother who also has been suffering from erythrocytosis. Mild bronchoplumonary dysplasia with frequent pulmonary infections during the first 6 years of life. The mother and an older sister (25 yrs.) were also affected by erythrocytosis, without PAH. She was thought to have polycythaemia vera. Phlebotomy treatment of the mother worsened symptoms. One older healthy brother is 28 yrs old. Two siblings died in the newborn age (a brother at day 5 due to a suspected congenital heart defect, a sister at day 14 with multi-organ failure due to multiple thromboses).
Examination findings	No pathologic findings apart from discrete plethora. Normal spleen size.

Laboratory parameters	Hb (g/L)	Hct (%)	RBC (x10^{12}/L)	MCV (fL)	WBC (× 10^9/L)	Pts (× 10^9/L)	Bone marrow	EPO (mU/ml)
	192	53	6.0	88	6.2	246	Normocellular, increased erythropoiesis, no dysplasia	9.64 (nl. 6-23)
	Soluble transferrin receptor 5.9 mg/l (nl. 1.9 – 4.4). Normal blood gas analysis. Calculated P50 27 mmHg.							
Molecular diagnosis	*VHL* and the EPOR gene wild-type in a sequencing analysis at age 9. Sequencing of the EPAS1 gene encoding HIF2αrevealed a heterozygous missense mutation: c.1609G>C, p.Gly537Arg, (G537R). The mother and the affected sister were also heterozygous for this mutation. It affects a different nucleotide but leads to the same aa change as described in the first case of congenital erythrocytosis due to a HIF2α mutation. The present case is inherited as an autosomal dominant disorder.							
Clinical follow-up and events	Treatment with low dose aspirin was continued. In addition, the patient has been treated with the phosphodiesterase inhibitor sildenafil. Treatment was initiated prior to the molecular diagnosis. It led to an improvement of the clinical status and to stabilization of the echocardiographic findings.							

Case 16 – Idiopathic erythrocytosis with elevated serum erythropoietin

Holger Cario
Dept. of Pediatrics and Adolescent Medicine, University Medical Center Ulm, Germany.

Clinical presentation	A 31 year old woman at presentation was diagnosed with erythrocytosis at the occasion of an acute infection at age 4. At present no symptoms, no pathologic signs and features.
Patient and family history	During childhood haematocrit was constantly increasing. Maximum haematocrit at age 14 with 0.78 (Hb 24.1 g/dl, see below) associated with low platelet count. There were never severe hyperviscosity symptoms. Single phlebotomies were each followed by immediate increase of the haematocrit to pre-phlebotomy levels. From age 14 to 16 treatment with enalapril lowered the haematocrit to a minimum of 61 with subsequent increase to 65 percent. From age 16 to 20 treatment with losartan was given. Similar course as with enalapril, i.e. initial haematocrit response and later increase to up to 70 percent. At the age of 20 years six serial phlebotomies of 500 ml within 14 weeks. Thereafter stabilization of the haematocrit in the range for 52-55 percent. At age 26 two single serial phlebotomies, one during pregnancy at age 27, one several month after first delivery. Since that time the haematocrit has been stable. Two successful pregnancies at age 27 and 29, healthy children. Family history without any evidence of other affected individuals.
Examination findings	No pathologic findings found apart from discrete plethora. The spleen size was normal. Echocardiography, abdominal ultrasound, previous CNS MRI (in adolescence), pulmonary function tests were all without pathologic findings.

Laboratory parameters	Hb (g/L)	Hct (%)	RBC (x10^{12}/L)	MCV (fL)	WBC (×10^9/L)	Pts (×10^9/L)	Bone marrow	EPO (mU/ml)
	Highest (at age 14):							
	241	78	8.6	78	6.2	122	increased erythropoiesis, no dysplasia	69 (nl. 6 -20)
	Current (at age 31):							
	165	52	7.7	68	5.4	239		62 (nl. 3.3 -15.8)
	Other: Soluble transferrin receptor 18.69 mg/l (nl. 1.9 – 4.4). Normal blood gas analysis. Normal P50. Primary hypothyroidism (autoimmune thyroiditis), with thyroxine replacement instituted. Normal hormonal profile. Exclusion of causes for acquired secondary erythrocytosis including pulmonary function tests, echocardiography, MRI, methionine PET, angiography including stepwise blood sampling from both renal veins for EPO analysis. Exclusion of increased hepatic EPO synthesis by biopsy and EPO mRNA analysis.							
Molecular diagnosis	Sequencing of all known candidate genes including *EPO, VHL, EGLN 1,2*, and *3, EPAS 1, HIF1A*, and others failed to detect a single mutation causing the presumably congenital erythrocytosis in this patient.							
Clinical follow-up and events	Treatment with low dose aspirin during long-distance flights. Treatment with low molecular heparin during pregnancy. Phlebotomies as mentioned above, performed if haematocrit increases above 55%. No regular treatment. Last echocardiography excluded pulmonary hypertension							

Case 17 – congenital erythrocytosis without molecular diagnosis

Susana Rives

Dept. of Pediatric Hematology and Oncology, Hospital Sant Joan de Déu de Barcelona, University of Barcelona, Spain.

Clinical presentation

A 15-year old female, with erythrocytosis diagnosed at age 5 detected in blood analysis performed due to hypercholesterolemia. The girl was asymptomatic at diagnosis.

Patient and family history

Familial hypercholesterolemia. No other relevant diseases. Parents and sister with normal red blood cell parameters.
- Father (35-yr) → RBC 4.8 x 10^{12}/L, Hb 149 g/L, Hct 0.44, MCV 91
- Mother (34-yr) → RBC 4.86 x 1012/L, Hb 131 g/L, Hct 0.44, MCV 88
- Sister (15- months) → RBC 4.43 x 1012/L, Hb 124 g/L, Hct 0.37, MCV 82

Examination findings

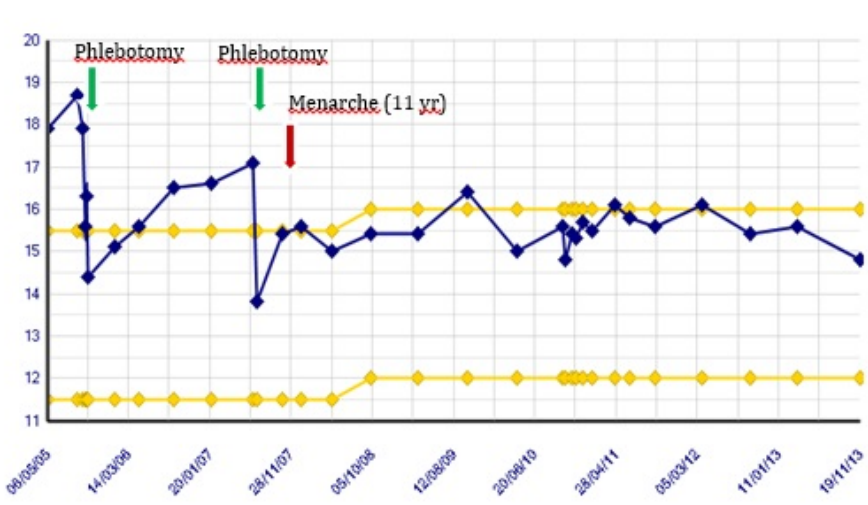
There were no abnormal findings on examination except for mild plethora. No splenomegaly.

Laboratory parameters

Age 5

Hb (g/L)	Hct (%)	RBC (x10^{12}/L)	MCV (fL)	WBC (×10^9/L)	Pts (×10^9/L)	Bone marrow	EPO (mU/ml)
178	55	6.67	82	7.9	249	Normocellular, mild increase of erythropoiesis	12.8 (nl. 4-20)

Other:
– Ferritin 26 microgram/L (normal)
– Hb HPLC: HbA_2 2.5%, HbF 0.5%. No Hb variants
– O_2 saturation: 98% (room air, pulse oximeter)
– Haemoglobin affinity for Oxygen: normal p50
– Endogenous erythroid colonies (EEC) assay
No endogenous growth of erythroid colonies: BFU-E (with EPO 120, VC 12.5 +/-7, w/o EPO: 0)
Red Cell Volume test (nuclear scan) : 31.8 mL/Kg. High normal reference for her age and sex: 24.7 mL/Kg.

Molecular diagnosis	– *HUMARA* gene: no clonality – PRV-1 quantification (granulocytes RNA): 0.14 (normal 0-15) – No mutations found in the following genes: *EPOR* gene, *VHL, HIF2A* (exon 12), *JAK-2, PHD2* (P317 and R371).
Clinical follow-up and events	The patient remained asymptomatic most of the time, mild headaches and occasional aquagenic pruritus with Hb ranging from 170 to 187 g/L (9 yr old). Treatment with phlebotomy was performed in 2 occasions (at age 9 and 11 years): 300 mL (12% blood volume) per week x 3 weeks. Menarche took place at age 12 years. Since then, the Hb values were from 148 to 163 g/L without phlebotomy and normal ferritin (20-30 microgram/L) without symptoms. At age 15 years she had a breast fibroadenoma. Evolution Hb (g/dL) 

Case 18 – Secondary erythrocytosis with a rare etiology

Tabita Magalhães Maia, Celeste Bento, M. Leticia Ribeiro
Serv. Hematologia Clínica, Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal.

Clinical Presentation

68 year old male patient, followed in an Hematology out-patient clinic for 10 years, with erythrocytosis, diagnosed as a polycythemia vera negative patient, under monthly phlebotomies. Came to our consultation for a second opinion, has he was tired of being phlebotomized and was searching for a different treatment option.

Patient and family History

No history of thrombosis, no smoker, no constitutional symptoms, no known previous diseases, no chronic medication, besides aspirin, and no family history of erythrocytosis.

Examination Findings

No facial plethora, body mass index of 24, normal heart and lung auscultation. Abdominal examination: a palpable mass of 10 cm in the upper left quadrant, that projected to the median abdominal line (spleen? other mass?).

Laboratory parameters

	Hb (g/L)	Hct (%)	RBC (x10^{12}/L)	MCV (fL)	WBC (× 10^9/L)	Pts (× 10^9/L)	EPO (mU/ml)
First consultation*	170	52.0	6.03	82.7	5.2	231	32

*(45 Days after flebotomy)
Normal kidney and liver function tests (Urea, creatinine, transaminases and DHL)

Molecular diagnosis

The patient had already been extensively studied in the molecular setting (*JAK2* V617F and Exon 12 mutations, *HBB, HBA, VHL, HIF, PHD2, BPGM* and *EPOR*).

Clinical follow-up and events

In order to elucidate the abdominal mass we preformed an abdominal ultrasound followed by a CT abdominal scan that showed a left kidney with a longitudinal diameter of 22 cm, totally composed of cysts, without visualization of renal parenchyma. The cysts had different dimensions (from 1 cm to 6 cm) and the majority were filled with liquid. The right kidney presented normal dimensions and structure. The absence of function of the left kidney was proved by a renal scintigraphy. Based on these findings he was diagnosed with a very rare condition: Unilateral polycystic kidney disease (UPKD). There are some cases described in the literature that show a clear association of this disorder with secondary erythrocytosis and the treatment is nephrectomy. After nephrology and urology consultations, the patient had his left kidney removed. EPO level of the intra-cystic liquid of the kidney was very high (234 mU/ml). 3 months after nephrectomy he had normal hemoglobin values, without phlebotomy, and now, one year later, he maintains normal hemoglobin levels, without any medical intervention.

	Hb (g/L)	Hct (%)	RBC (x10^{12}/L)	MCV (fL)	WBC (× 10^9/L)	Pts (× 10^9/L)	EPO (mU/ml)
Before nephrectomy	183	56.5	6.50	86.9	7.49	237	N/A
3 months after nephrectomy	144	41.2	5.10	89.1	6.2	330	20
1 year after nephrectomy	140	40.2	5.13	88.9	7.1	251	22

Case 19 – Not everything that shines is gold

Tabita Magalhães Maia, Helena Almeida, Celeste Bento, M. Leticia Ribeiro
Serv. Hematologia Clínica, Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal.

Clinical presentation

31 year old male patient with erythrocytosis detected in a routine clinical analysis.

Patient and family history

Previously healthy, moderate smoker, no pulmonary or cardiac symptoms, Body mass index of 28. Came to our consultation due to hemoglobin of 185 g/L, hematocrit of 55%. When asked he referred that he has always had this hemoglobin levels. His 2 sisters and his father are hematologically normal, and his mother had a hemoglobin of 158 g/L, hematocrit of 48 (and no known possible cause).

Examination findings

Normal physical examination.
Normal renal, abdominal and cardiac ultrasound. Normal respiratory function tests.

Laboratory parameters

	Hb (g/L)	Hct (%)	RBC (x10^{12}/L)	MCV (fL)	WBC (× 10^9/L)	Pts (× 10^9/L)	EPO (mU /ml)
3 years before consultation	184	54.2	5.8	93.0	8.4	395	N/A
First consultation (hematology)	185	55.0	6.0	89.9	10.2	360	12
One year after1st consultation	183	54.0	5.76	92.1	7.5	285	N/A
Two years after 1st consultation	186	56.2	6.2	88.3	7.8	320	20

Molecular diagnosis

Since we were thinking of an autosomal dominant disorder, with normal EPO levels, we started by performing a hemoglobin study by HPLC which was normal (AA2 without any atypical band and 2.8% of Hb A2, Hb F 0.8%). Because some high affinity hemoglobin variants don't show in the HPLC, we preformed the *HBB* and *HBA* sequencing and no mutations were identified. We then sequenced the *PHD2* gene (also with mutations associated with autosomal dominant erythrocytosis), and we found a c.380G>C (Cys127Ser) mutation. His mother had the same mutation. Even though both have erythrocytosis and the same mutation, the truth is that this was described as a polymorphism, and it is not expected to be (alone) the cause of this patient' erythrocytosis. We then studied all the other known genes associated with erythrocytosis in the *propositus* and his mother (*JAK2* V617F and exon 12, *EPOR, VHL, HIF* and *BPGM*) but no mutations were found.

Clinical follow-up and events

Our *propositus*, in the last consultation, started phlebotomies, and his mother has maintained a stable hemoglobin level, without treatment. Both are under aspirin, without thromboembolic events. Even though all the known genes associated with erythrocytosis are all studied, and no evidence of secondary acquired causes, there is a clear family history, and it is important to clarify if this polymorphism along with other mutation can cause the disease.

II. HEREDITARY THROMBOCYTOSIS

Chapter 1
Thrombocytosis: A general overview

Sylvie Hermouet, Eric Lippert

University Hospital of Nantes, France; University Hospital Bordeaux, France

The definition of thrombocytosis is a platelet count in blood above 450 x 10^9/L. Here we wish to discuss chronic thrombocytosis, characterized by a platelet count above 450 x 10^9 / L for at least 6 months. Although thrombocythemia and thrombocytosis have the same meaning, it has become usual to use thrombocythemia (rather than thrombocytosis) in the context of essential thrombocythemia (ET), one of the myeloproliferative neoplasms (MPNs) characterized by chronic thrombocytosis.

Different types of chronic thrombocytosis (primary or secondary, familial / hereditary or sporadic) and their complications (thrombosis, myelofibrosis, malignant transformation and more rarely, bleeding) have been known for a long time, and the most frequent causes of secondary thrombocytosis (inflammation, iron deficiency) are now well identified.

A primary thrombocytosis is a thrombocytosis caused by an alteration in hematopoietic cells; the serum level of thrombopoietin (TPO), the main cytokine responsible for the production of

platelets, is low or normal. Primary thrombocytosis can be hereditary (rare) or acquired (more frequent, in the context of MPNs and myelodysplastic syndromes (MDS). In MDS with thombocytosis, abnormalities of chromosomes 5 or 3 are frequently found. A provisional entity defined by the World Health Organization (WHO), refractory anemia with ring sideroblasts and thrombocytosis (RARS-T) displays criteria of both MPNs and MDS.

A secondary thrombocytosis is a thrombocytosis due to an external cause; the serum level of TPO is frequently elevated. Secondary thrombocytosis is usually acquired: ailments resulting in elevated platelet counts include inflammation, iron deficiency, and asplenia (absence of spleen). In rare cases, secondary thrombocytosis is hereditary (familial).

A hereditary thrombocytosis (HT) may be primary or secondary. By definition HT is due to a genetic alteration that can be transmitted to offspring – i.e. it is associated with a familial, or hereditary, genetic cause.

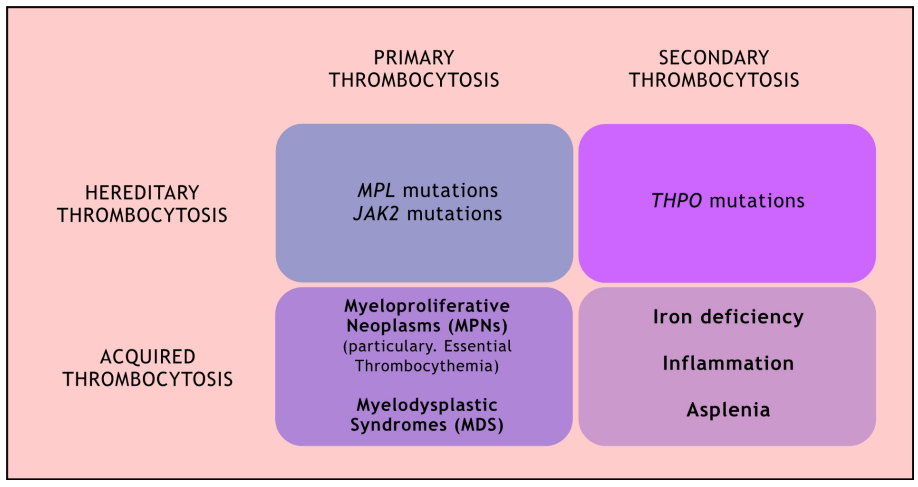


Figure II.1 Classification of the different types of chronic thrombocytosis

It has now been recognized for more than 50 years that certain patients may present with seemingly hereditary (familial) thrombocytosis (Beretta Anguissola A and Prato V. 1961). Such patients occasionally suffered from complications ranging from thrombosis (Spach MS *et al*., 1963) to blastic transformation (Fickers M *et al*., 1974). However, almost 40 years passed prior to the discovery of the first germline mutation associated with HT in *THPO*, the gene encoding TPO (Wiestner A *et al*., 1998). New mutations also associated with HT were later identified in *MPL*, the gene that encodes the receptor for TPO (i.e. *MPL* S505N) (Ding J *et al*., 2004). In the following years, ET (one of the MPNs) was found to be associated with somatic mutations in 3 genes: mainly the V617F mutation in the *JAK2* gene, in about 60-70% of cases (James C *et al*., 2005); mutations in the *CALR* (calreticulin) gene, in about 25-30% of cases (Klampfl T *et al*., 2013); and W515L/K/A/R mutations in the *MPL* gene, in 5% of cases (Pikman Y *et al*., 2006). *MPL*W515L/K/A/R mutations were also detected in a minority (<10%) of patients diagnosed with primary myelofibrosis (PMF), the more severe form of MPNs.

The finding that both HT and ET could be associated with mutations in the same gene (*MPL*) led to a new pathogenic model which stated that different defects in the *MPL* gene could lead to distinct forms of chronic thrombocytosis, either benign in the case of germline mutation -in the context of HT- or malignant in the case of somatic mutation -in the context of ET and PMF (Skoda RC. 2009). The validity of this model was confirmed by the discovery in the context of HT of several germline mutations affecting the *JAK2* gene (Mead AJ *et al*., 2012; Marty C *et al*., 2014). Moreover, it is important to note that all the HT-causing mutations identified so far involve three genes which encode the molecules that are most important for the production of megakaryocytes and platelets: TPO; MPL, the receptor for TPO; and JAK2, the tyrosine-kinase coupled to MPL that transmits the proliferation and differentiation signals of TPO.

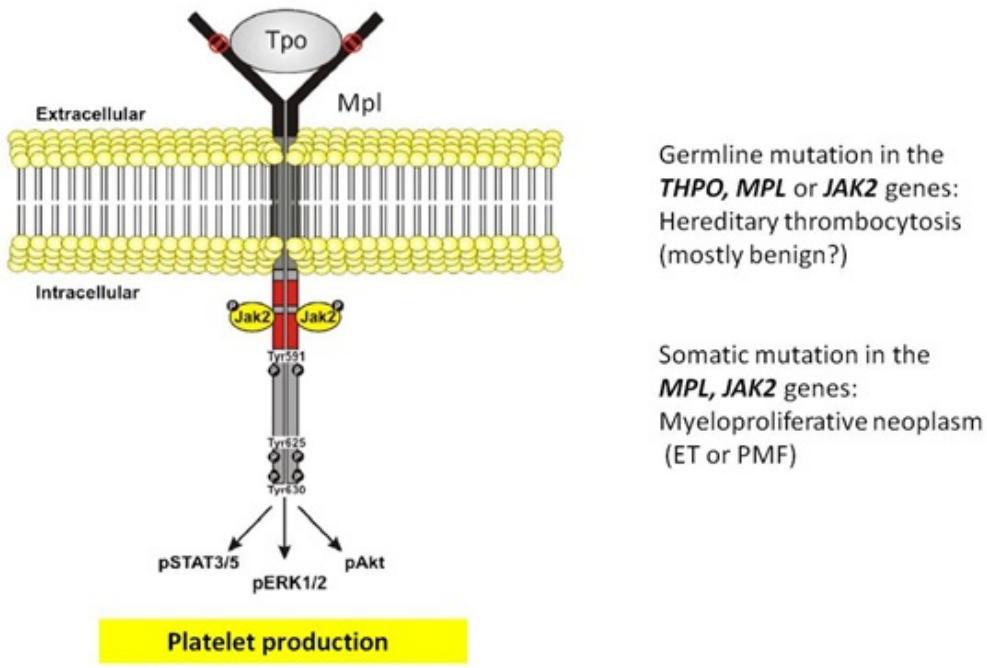


Figure II.2 Representation of TPO and associated signaling molecules encoded by the genes found mutated in the context of HT (*THPO, MPL, JAK2*) and MPNs (*MPL, JAK2*) (*scheme made after Geddis AE. Semin Hematol 2010*)

The following pages and chapters present in more detail the latest knowledge on HT, with the aim to facilitate the diagnosis of this rare disease.

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Chapter 2
Pathophysisology of hereditary thrombocytoses

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In the past ten years major progress has been made in understanding the biology of HT, and this chapter aims to describe and discuss the main HT-causing mutations presently known. First of all, one should consider screening for germline, HT-causing mutations those patients with:

* Platelet counts > 450 x10^9/L

AND

* No essential thrombocythemia (no somatic *JAK2*V617F mutation, no calreticulin (CALR) exon 9 mutation.

AND

* No identified cause of secondary thrombocytosis: inflammation, iron deficiency, asplenia.

No myelodysplastic syndrome and no acute myeloid leukemia (bone marrow biopsy or aspiration; karyotype)

* Young patients

* Patients with a positive family history

Depending on the mutated gene, HT is primary or secondary. Primary HT is due to genetic alterations that affect mainly haematopoietic progenitors; since the mutation affects all cells, it is a polyclonal disease. Secondary HT is also a polyclonal disease, but the causes are external to haematopoietic progenitors. We now know that different germline mutations in three main genes, *THPO, MPL* and *JAK2*, can cause HT (Kikuchi M *et al*., 1995; Hong WJ and Gotlib J, 2014). The genes affected encode either TPO, the cytokine required for megakaryocyte and platelet production, or signaling molecules critical for the signaling of TPO, as follows:

– Germline mutations in *THPO*, the gene encoding TPO, lead to secondary thrombocytosis (with high TPO level in blood).

– Germline mutations in *MPL*, the gene encoding the receptor for TPO, expressed by hematopoietic progenitors and platelets, lead to primary thrombocytosis (TPO level in blood is usually low or normal).

– Germline mutations in *JAK2*, the gene encoding the MPL-coupled tyrosine-kinase that transmits TPO signaling, also lead to primary thrombocytosis (with low or normal TPO level in blood).

However, not all HT patients carry mutations in the *THPO, MPL*, or *JAK2* genes (Wiestner A *et al*., 2000) and it is likely that HT can also be caused by alterations in other genes. For instance, one can imagine that mutations involved in HT may involve genes which encode cytokines, signaling molecules, cytoskeleton-associated molecules or other membrane proteins important for

platelet adhesion or aggregation which would result in functionally deficient platelets and consequently, chronically increased platelet production (thrombocytosis) in an effort to compensate for the platelet defects.

Table II.1 Overview of HT-causing mutations

Gene	Product	Type of mutation	Function	TPO level	Type of HT
THPO	TPO	Promoter Intron/exon junction	Increased translation and expression of THPO, stimulation of the production of megakaryocytes and platelets	High	Secondary
MPL	MPL (receptor for TPO)	Gain-of-function	Increased transmission of the proliferation and differentiation signals of TPO Regulation of the level of TPO circulating in blood	Low or normal	Primary
JAK2	JAK2 (Mpl-coupled tyrosine kinase)	Gain-of-function	Increased transmission of TPO/MPL signals Regulation of MPL maturation and localization at the surface of platelet membrane	Low or normal	Primary
Others	To be identified	Loss-of-function?	Maintenance of adequate structure and function of platelet membranes, notably in regard of MPL surface expression and platelet adhesion and aggregation	Expected to be high	Secondary

2.1 Germline *THPO* mutations

TPO is the main cytokine involved in the production of megakaryocytes and platelets. TPO also plays a critical role on the maintenance and fate of early myeloid progenitors. Therefore it was

logical that linkage disequilibrium studies allowed the discovery of mutations in the *THPO* gene, which encodes TPO, in several families with chronic thrombocytosis.

The *THPO* mutations found in families with thrombocytosis (see below) were all located either in the 5'UTR of the mRNA or at exon/intron boundaries, resulting in defective alternative splicing.

Published *THPO* mutations:

- G>C transition in the splice donor of intron 3 of *THPO* (Schlemper RJ *et al*., 1994; Wiestner A *et al*., 1998; Liu K *et al*., 2008);
- G>T transversion at position 516 (G516T) of the 5'-untranslated region (5'-UTR) of the TPO mRNA (Kikuchi M *et al*., 1995; Ghilardi N *et al*., 1999a);
- 1-base pair G deletion in the 5' untranslated region at nucleotide position 3252 and localisation of deleted G 47 bases upstream of the authentic initiation codon (Kondo T *et al*., 1998);
- A>G mutation in intron 3 (Jorgensen MJ *et al*., 1998);
- G185T *THPO* mutation in the 5' UTR region (Graziano C *et al*., 2009);
- T>C mutation in intron 2 (Zhang B *et al*., 2011);
- c.13+1 G>C mutation in splice donor site of intron 3 of *THPO* (Stockklausner C *et al*., 2012).

In vitro studies proved that these types of mutations increase the effectiveness of TPO translation (Ghilardi N *et al*., 1998; Ghilardi N *et al*., 1999b).

In normal subjects, upstream of the start codon for *THPO*, alternative ATG encode small uORF, the translation of which impairs TPO translation. All mutations found in the *THPO* gene so far result in alteration of these uORF, thus lifting the repression on TPO translation, leading to increased expression of TPO, high level of TPO in peripheral blood, and increased production of platelets.

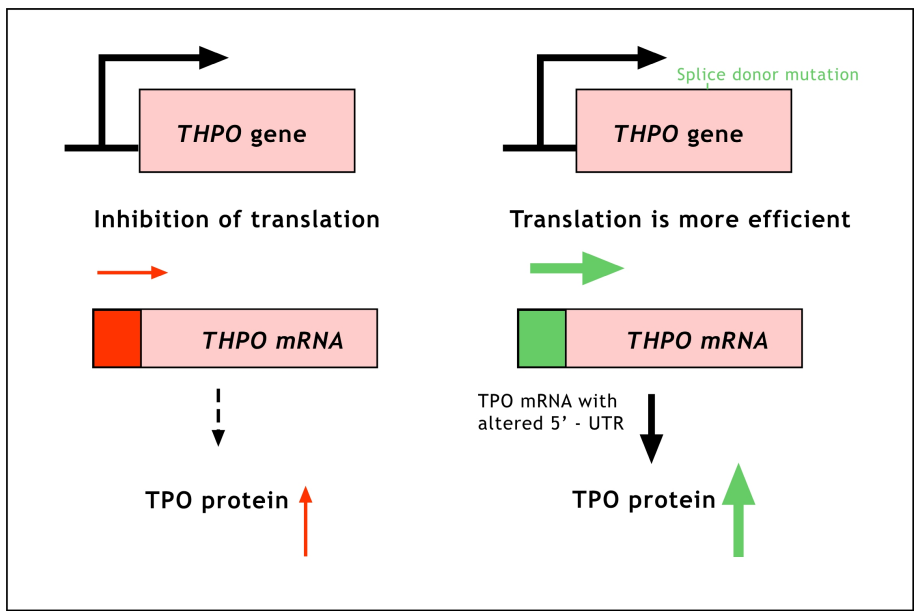

Figure II.3 *THPO* mutations in hereditary thrombocytosis
(*After Wiestner et al. Nat Genet. 1998; 18:49-52*)

Different clinical phenotypes have been described in families with *THPO* mutations, ranging from isolated thrombocytosis to thrombocytosis associated with limb defects (Graziano C *et al*., 2009). Reported complications include thrombotic events, myelofibrosis, and acute leukemia. These aspects are described and discussed in Chapter 3.

2.2 Germline MPL mutations

The discovery and analysis of *MPL* mutations has helped decipher the physiological regulation of platelet homeostasis, notably the role played by the MPL receptor in the regulation of the level of free TPO in blood. Indeed the platelet number in blood is maintained by a negative feedback loop which involves the clearance of TPO by MPL receptors expressed at the surface of platelets (reviewed by Skoda RC, 2009, 2010, 2014). Thus a low expression of MPL either due to low platelet counts, or reduced expression of MPL at the platelet surface (even in the context of thrombocytosis) may result in increased circulating TPO and subsequently, increased stimulation of megakaryocyto-poiesis and platelet production. This important mechanism of regulation of platelet counts is represented in the next figure.

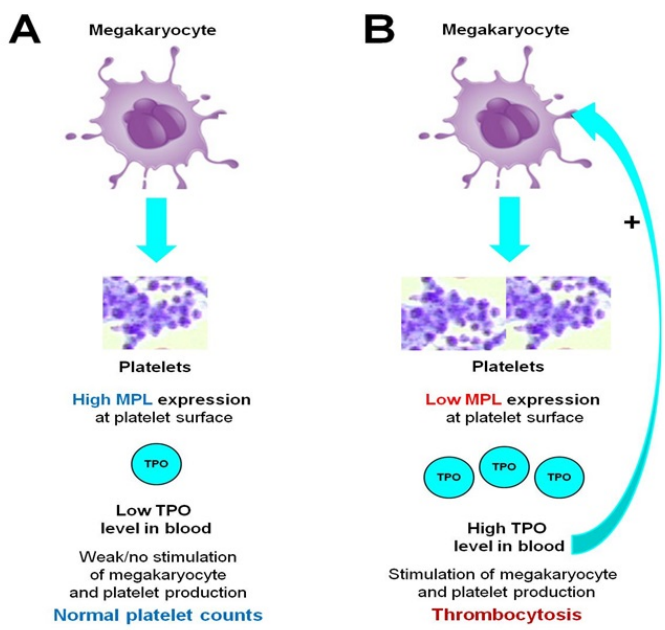

Figure II.4 Regulation of TPO level and platelet production by platelet MPL expression

A: A normal (high) expression of MPL at platelet surface keeps the TPO level in blood within normal values, leading to normal megakaryocytopoiesis and normal platelet counts. B: A low number of platelets or/and a low or absent expression of MPL at platelet surface, do not allow normal clearance of blood TPO. The TPO level in serum increases, which leads to TPO-induced stimulation of megakaryocytopoiesis and increased production of platelets (thrombocytosis).

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Inherited alteration of the receptor for TPO (MPL) has been shown to result in thrombocytosis with familial presentation. In such cases, the genetic alterations may either be a single nucleotide polymorphism (SNP) restricted to specific ethnic groups, such as African Americans (Moliterno AR *et al*., 2004) or Arabic populations (El Harith el-HA *et al*., 2009), or a bona fide mutation, such as the S505N mutation (Ding J *et al*., 2004). Recently, the activating *MPL*W515R mutation initially discovered as a somatic mutation in the context of MPNs was also detected as a germline mutation in the context of HT (Broseus J *et al*., 2010; Vilaine M *et al*., 2012). Of note, *MPL* W515 mutations are mostly found in acquired MPNs, where they are detected in 5-10% of PMF and 1-5% of ET cases (Pikman Y *et al*., 2006; Pardanani AD *et al*., 2006). Interestingly, the *MPL* S505N and W515L mutations were reported associated and present in the same cells in one MPN patient (Boyd EM *et al*., 2010).

In the context of HT, *MPL* mutations typically result in a gain-of-function of the MPL receptor and consequently, in increased response to TPO stimulation and enhanced platelet production. Logically, most patients with germline mutations in the *MPL* gene present with a low or normal level of TPO in blood. However, this is not true for individuals homozygous for the *MPL* P106L mutation, who have a high TPO level in blood. The figure and table below present the germline *MPL* mutations reported so far in HT, as well as their localization.

Table II.2 Main HT-associated *MPL* mutations

Germline *MPL* genetic alteration	Exon Nbr	Codon	SNP / Mutation	References
c.117G>T	2	**K39N**	SNP rs17292650 ≥ 7% African Americans	Moliterno AR *et al.*, 2004
c.317G>T	3	**P106L**	Mutation 3.3% Arab population	El-Harith el-HA *et al.*, 2009; Stockklausner C *et al.*, 2012
c.754T>C	5	**Y252H***	Mutation	Lambert MP *et al.*, 2012
c.1073G>A	10	**S505N**	Mutation	Ding J *et al.*, 2004; Liu K *et al.*, 2009; Teofili L *et al.*, 2010
c.1543T>C	10	**W515R**	Mutation	Broseus J *et al.*, 2010; Vilaine M *et al.*, 2012

(*) In this paediatric case, the 3 year old patient also carried the K39N SNP; the germline nature of the genetic alterations was not demonstrated.

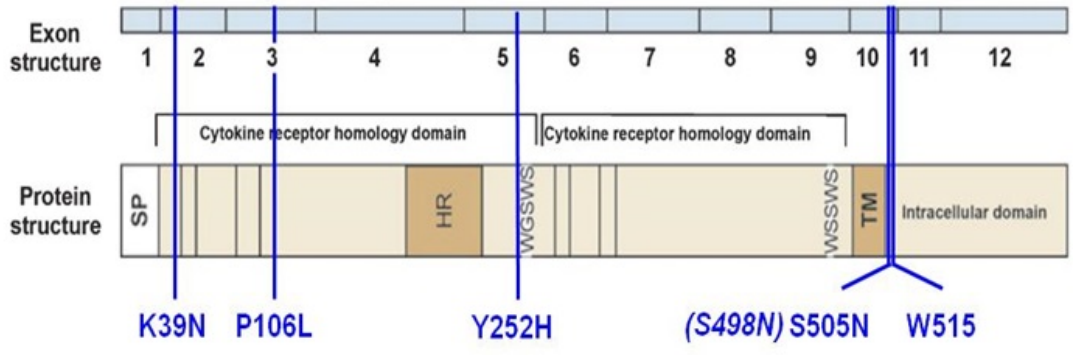

Figure II.5 Localisation of the main HT-associated *MPL* mutations
Adapted from Ballmaier & Germeshausen. Br J Haematol. 2009; 146(1):3.

The next figure represents the MPL receptor. The MPL receptor exists as a homodimer, coupled mainly to the tyrosine kinase JAK2. MPL receptors can also couple to and activate TYK2, another tyrosine kinase of the JAK family. Upon binding of TPO, a change of conformation occurs which brings the two MPL chains closer together (dimerization of the MPL receptor), activates JAK2 (or TYK2) and induces the subsequent phosphorylation on tyrosine residues and activation of several STAT molecules (STAT3, STAT5, STAT6). Upon TPO binding, the MPL receptor can induce the activation and nuclear localization of STAT molecules, as well as the activation of the ERK-1/2 and AKT signaling pathways (Hitchcock IS, Kaushansky K., 2014). The localization of different MPL mutants discovered in HT is also shown.

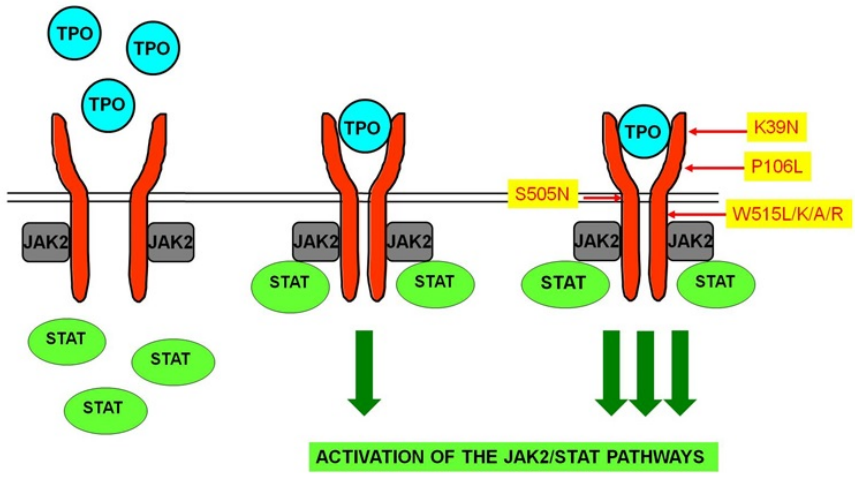

Figure II.6 The MPL receptor and location of different *MPL* mutations

Different mechanisms of action leading to activation of the MPL/JAK2/STAT pathways following stimulation by TPO have been described for HT-associated *MPL* mutations. The differences in the mode of action of *MPL* mutations depend essentially on the location of the mutated amino-acid.

– Mode of action of *MPL* K39N and P106L: Located in the extra-cellular domain of the MPL receptor, these SNP (K39N) and mutation (P106L) are expected to affect negatively the binding of TPO and MPL dimerization, with potential consequences on both TPO clearance in peripheral blood (less TPO clearance, high TPO level in blood) and MPL signaling (expected to be less efficient). Thus it is difficult to explain the thrombocytosis observed in homozygous

individuals. Moreover, individuals heterozygous for *MPL* K39N present with thrombocytosis, whereas homozygosity is required for *MPL* P106L carriers to develop thrombocytosis.

In vitro studies in transfected cell lines concluded that both mutations resulted in increased TPO signaling and cytokine independence (Moliterno AR *et al*., 2004; El-Harith el-HA *et al*., 2009; Stockklausner C *et al*., 2012). Moreover, an impaired glycosylation and low expression at platelet cell surface was reported for the MPL K39N mutant (Moliterno AR *et al*., 2004). Similarly, the P106L mutation was recently shown to lead to impaired glycosylation and lack of membrane localization of the MPL receptor (Stockklausner C *et al*., 2015). Consistently, the TPO serum level was found to be markedly elevated in patients with homozygous *MPL* P106L mutation, and normal in heterozygous individuals (Stockklausner C *et al*., 2015). Only the individuals homozygous for *MPL* P106L present with thrombocytosis.

The main mechanism responsible for the stimulation of platelet production in patients with *MPL* K39N or P106L mutation seems to be a feedback loop subsequent to the elevation of TPO serum levels triggered by the low expression of MPL receptors at the surface of platelets (figure next page). For heterozygous individuals, the presence of wild type MPL receptors is sufficient for a normal activation of downstream signaling in response to TPO, and the elevation of platelet counts reflects the level of TPO. In contrast, patients homozygous for the *MPL* K39N mutation or the *MPL* P106L mutation have a very high TPO level in blood but little or no MPL at the membrane surface.

Of note, it is unclear that MPL P106L binds TPO (to our knowledge, this has not been investigated).

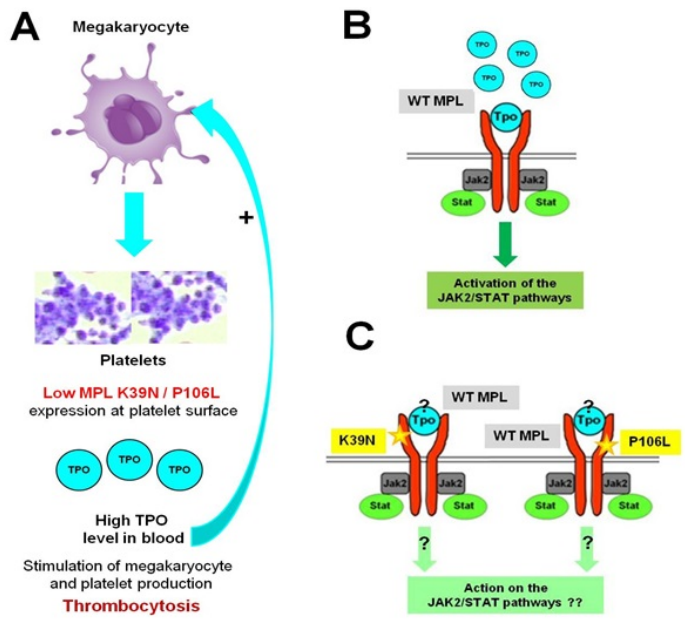

Figure II.7 Putative mechanism of action of the *MPL* K39N and P106L mutations A: A low expression of mutant MPL at platelet surface does not allow sufficient clearance of TPO and the TPO level in blood serum increases, which leads to increased TPO stimulation of megakaryocyte and platelet production (thrombocytosis). B: Representation of increased TPO-activation of the JAK2/STAT pathways via the wild-type (WT) MPL receptors. C: The efficacy of TPO binding to heterodimeric K39N/WT and P106L/WT MPL receptors and subsequent activation of the JAK2/STAT pathways in heterozygous individuals is unknown.

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If mutant MPL P106L receptors do not bind TPO, either for structural reasons or for lack of expression at cell or platelet surface, one can propose two main hypotheses to explain thrombocytosis in patients with homozygous MPL P106L mutation:

1. One hypothesis is that the P106L mutation stabilizes the MPL dimer in the absence of TPO. Thus MPL P106L homodimers are constantly in an activated conformation, bound to JAK2 and therefore, activation of the JAK2/STAT pathways is constant. Since MPL P106L is not expressed at the membrane surface, it is postulated that JAK2-coupled MPL P106L homodimeric receptors activate the STAT pathways inside the endoplasmic reticulum (Stockklausner C *et al.*, 2015).

2. Another hypothesis is that MPL P106L receptors do not transmit signals. For homozygous *MPL* P106L-mutated individuals, platelet production is then under the control of interleukin-6 (IL-6). Indeed it is established that TPO is not required for the production of platelets: TPO can be replaced by IL-6 in this function and a high level of TPO (here due to the lack of MPL expression on platelets) is known to strongly stimulate the production of IL-6 by the liver, as represented in the next figure.

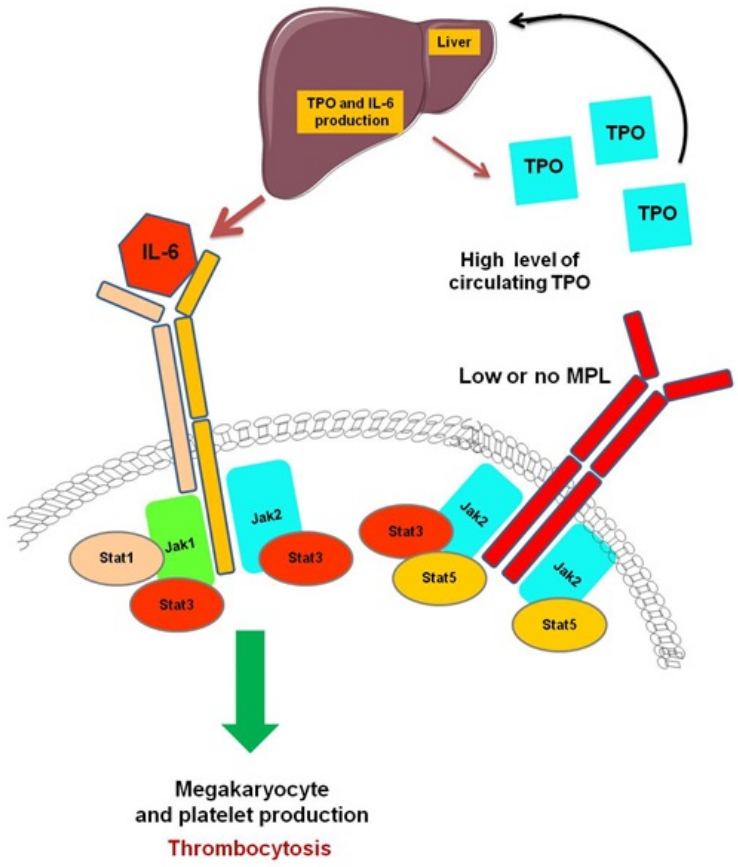

Figure II.8 Platelet production regulated by TPO-induced IL-6 in case of absence of MPL

In order to prove or disprove this hypothesis, the blood level of IL-6 needs to be measured in patients found to be heterozygous or homozygous for the *MPL* P106L and K39N mutations.

– Mode of action of the *MPL*S505N mutation: Located in the trans-membrane domain of the MPL receptor, the S505N mutation is expected to affect the formation of MPL dimers necessary for optimal activation of MPL and JAK2 after TPO binding. Indeed, the S505N mutation has been shown to induce autonomous dimerization of MPL homodimers and thus, enhanced activation of the JAK2/STAT pathways, including in the absence of TPO (Ding J *et al*., 2009). It is also established that the S505N mutation results in increased activation of the MPL/JAK2/STAT pathways (Ding J *et al*., 2009; Stockklausner C *et al*., 2015). Thus the strong aminoacid polarity of the S505N mutant and subsequent TPO-independent dimerization of MPL S505N dimers explain the increased activation of the JAK2/STAT pathways in individuals with S505N mutation. Similar observations had been made for the S498N mutation, also located in the trans-membrane region: the S498N mutation abrogated factor-dependency in interleukin-3-dependent murine cell lines, and the mutant MPL S498N protein constitutively activated the JAK2-STAT3/STAT5 pathways in the same cell lines (Onishi M *et al*., 1996). Consistently, a heterozygous status for the *MPL* S505N mutation is sufficient to present with thrombocytosis, and the TPO level in peripheral blood is low.

– Mode of action of *MPL*W515 mutations: Located in the intra-cellular domain of MPL close to the membrane, it is assumed that W515 mutations facilitate and stabilize MPL dimerization. The results of computer simulation studies suggest that like S505, W515 is important in keeping the trans-membrane domain of MPL in its correct position within the membrane. Mutations at the S505 and W515 positions cause movement of the trans-membrane domain and alter the conformation of the intracellular domain (Lee T-S *et al*., 2011). Analysis *in vitro*, typically in transfected cell lines, showed that all mutations (L/K/A/R) of aminoacid W515 result in increased activation of the JAK2/STAT, ERK-1/2 and AKT pathways, both in absence and in presence of TPO (Pikman Y *et al*., 2006; unpublished personal observations).

MPL W515L/K/A/R mutations were first described as somatic mutations in the context of ET and PMF. However, we reported two members of a family who presented with an isolated,

moderate, thrombocytosis. These individuals were found to carry the *MPL* W515R mutation in a heterozygous fashion, and the germline nature of the *MPL* W515R mutation was established in this family (Broseus J *et al*., 2010; Vilaine M *et al*., 2012). The TPO level in blood is low in individuals with germline *MPL* W515R mutation. The presence of a *MPL* W515R mutation has been reported in a few other cases (all isolated cases) (Boyd EM *et al*., 2010).

As for germline *THPO* mutations, different clinical phenotypes and disease evolution have been described in families with germline *MPL* mutation. They range from isolated and stable thrombocytosis to various thrombotic events, myelofibrosis, or evolution toward acute leukemia (Teofili L *et al*., 2011; Giona F *et al*., 2012). The absence of thrombotic complications and importantly, the absence of evolution toward myelofibrosis in the family with germline *MPL* W515R mutation imply that alone, a *MPL*W515 mutation may not be sufficient to cause a MPN – additional mutations are required, in line with the classic multi-step process leading to cancer in humans. Moreover, it appears that certain individuals with germline *MPL* mutation are protected from acquiring such mutations. Uncovering the mechanisms that protect individuals with germline *MPL* W515R mutation from thrombosis and myelofibrosis will be very important to improve understanding of the pathogenesis of *MPL* W515-mutated myeloproliferative neoplasms.

Clinical aspects of *MPL*-mutated HT are further described and discussed in Chapter 3.

2.3 Germline *JAK2* mutations

JAK2 encodes a tyrosine kinase that is ubiquitously expressed and transmits the signaling of several cytokines (granulocyte colony stimulating growth factor (G-CSF), EPO, TPO, GM-CSF, IL-6 and other cytokines of the IL-6 family) as well as hormones, including the growth hormone (GH), luteinizing hormone (LH) and leptin. The best known mutation in the *JAK2* gene is the V617F mutation, a somatic mutation located in exon 14 of the *JAK2* gene, in the pseudokinase domain, and discovered in 2005 in blood cells of MPN patients (James C *et al*., 2005). The JAK2V617F mutation is found in 95-98% of patients with polycythaemia vera (PV), 60-70% of patients with ET, and

50% of patients with PMF. In haematopoietic progenitors the *JAK*2V617F mutation results in increased activation of JAK2 and the STAT5 pathways in response to cytokines with JAK2-coupled receptors, notably G-CSF, EPO and TPO. Consequently, presence of the *JAK2*V617F mutation leads to increased production of blood cells of all the myeloid lineages. Since 2005, other somatic, gain-of-function mutations have been discovered in the *JAK2* gene. These mutations were either associated with a generally benign form of chronic erythrocytosis (mutations in *JAK2* exon 12) or with pediatric B-cell acute lymphoid leukemia (the *JAK2*R683G/S and *JAK2*R867Q mutations, for instance) (Scott LM *et al*., 2007; Mullighan CG *et al*., 2009).

Unexpectedly, several germline *JAK2* mutations were recently discovered in the context of isolated familial thrombocytosis – i.e. individuals with a normal production of granulocytes and erythrocytes. HT-associated germline *JAK2* mutations are presented in the table below.

Table II.3 Germline *JAK2* mutations associated with HT

Germline *JAK2* mutation	Exon Nbr	Codon		References
c.1691G>A	13	**R564Q**	Autosomal dominant	Etheridge SL *et al.*, 2014
c.1822C>A	14	**H608N**	Autosomal dominant	Rumi E *et al.*, 2014
c.1894G>A	14	**V617I**	Autosomal dominant	Mead AJ *et al.*, 2012; Mead AJ *et al.*, 2013
c.2600G>A	20	**R867Q**	Autosomal dominant	Marty C *et al.*, 2014
c.2265T>A c.2813G>A	17 21	**S755R** **R938Q**	Autosomal dominant	Marty C *et al.*, 2014

The germline *JAK2* V617I mutation was identified first, in several members of a family with chronic thrombocytosis; importantly, *JAK2* V617I-affected patients had normal red and white blood cell counts (Mead AJ *et al*., 2012). In this family, individuals older than 40 years old suffered from various types of vascular events. Functional studies concluded that the *JAK2*V617I mutation was activating but only had a weak effect on JAK2/STAT5 signaling compared to the *JAK2*V617F mutation (Mead AJ *et al*., 2013). The absence of an effect on the granulocyte and erythroid lineages was not otherwise explained. Moreover, since JAK2 is ubiquitous but particularly abundant in blood cells and in the brain, and critical for the signaling of hormones such as GH and LH, one would expect a germline *JAK2* mutation to affect other cell systems in addition to blood cells, yet no such consequences were noted in the *JAK2*V617I family.

The discovery of the germline *JAK2*V617I mutation prompted a more careful analysis of the *JAK2* gene in unexplained cases of chronic thrombocytosis and several teams reported new germline mutations in the *JAK2* gene, all in residues other than V617. One, H608N, is also located in the pseudokinase domain; it was described as associated with isolated thrombocytosis (Rumi E *et al*., 2014). Another is the R564Q mutation (*JAK2*R564Q), also associated with isolated thrombocytosis (Etheridge SL *et al*., 2014). Interestingly, *JAK2*R564Q was shown to increase JAK2 kinase activity in a manner similar to the *JAK2*V617F mutation (Etheridge SL *et al*., 2014). Marty et al. reported a germline mutation in the JAK2 kinase domain (R867Q) in a family with isolated HT (Marty C *et al*., 2014). Of note, this mutation had previously been reported as a somatic mutation in the context of paediatric B-cell lymphoid acute leukaemia (B-ALL) (Mullighan CG *et al*., 2009). Marty C *et al*. also described the presence of two *in cis* mutations in the *JAK2* gene -on the same allele- in another HT family: one mutation was located in the pseudokinase domain (S755R), the other in the kinase domain (R948Q) (Marty C *et al*., 2014). Functional studies of the three *JAK2* mutants in the murine Ba/F3-MPL cell line revealed a higher expression of MPL at the cell surface, compared to *JAK2*V617F (Marty C *et al*., 2014).

Hence it appears that all the HT-associated germline *JAK2* mutations identified so far can activate the JAK2/STAT5 pathways, sometimes as efficiently as *JAK2*V617F. In addition, the ex-

pression level of MPL at the membrane surface may be higher with germline *JAK2* mutants than with *JAK2*V617F. Most patients present with isolated thrombocytosis; only one family suffered from vascular complications. Despite the fact that one of the germline *JAK2* mutations (R867Q) was also detected in a case of B-ALL, no evolution toward a MPN was reported in these families.

As with individuals with germline *MPL*W515R mutation, patients with germline *JAK2* mutation seem to be protected from thrombotic events, myelofibrosis or malignant transformation. The mechanisms of this protection are unknown. Moreover, the lack of effect of activating germline *JAK2* mutations on granulocytes and red blood cells, and the absence of hormonal and metabolic consequences, are additional mysteries.

2.4 Other HT-associated germline mutations

As described above, all the HT-associated mutations published so far act by activating a cascade of molecules that stimulate platelet production: the TPO/MPL/JAK2 axis. To this day there is only one exception: gelsolin.

Gelsolin is a protein that binds actin and is involved in actin filament assembly. Studies of gelsolin knockout (*GSN* KO) mice revealed that the absence of or deficiency in gelsolin led to abnormal neutrophil and platelet functions, with elevated leukocyte and neutrophil blood counts and a longer bleeding time, as well as a blunted response to inflammation. Platelet counts were not reported in this paper (Witke *et al*., 1995). Hence gelsolin appears to be an important -but not essential- molecule for optimal neutrophil and platelet function, with consequences on the response to inflammation.

Recently a germline mutation in the *GSN* gene was identified in a family with chronic thrombocytosis (Pianta A *et al*., 2013). Based on the information obtained with the *GSN* KO mice, one can envision that the HT-associated *GSN* mutation may alter platelet function and consequently, response to inflammation and haemostasis. In this context, increasing the production of platelets would be a logical effort to compensate for a functional defect.

More generally, the discovery of a *GSN* mutation in HT, together with the unexpected and poorly understood effects of germline *MPL*W515R and *JAK2* mutations (lack of effect in leukocytes, rarity of thrombotic events, absence of myelofibrosis or malignant transformation), suggests that HT and its complications may be the consequences of at least two mechanisms:

1. A germline gain-of-function mutation of the TPO/MPL/JAK2 axis, the main pathway responsible for the production of megakaryocytes and platelets. Note that TPO and TPO-binding MPL also regulate the level of production of IL-6, a cytokine that stimulates both thrombopoiesis and inflammation.

2. A germline loss-of-function mutation in platelets (or/and other blood elements) that are required for an adequate response to inflammation. In the context of HT which compensates for a loss-of-function mutation, one would expect the risks of thrombotic events, inflammatory disease, and progression toward malignancy to be low.

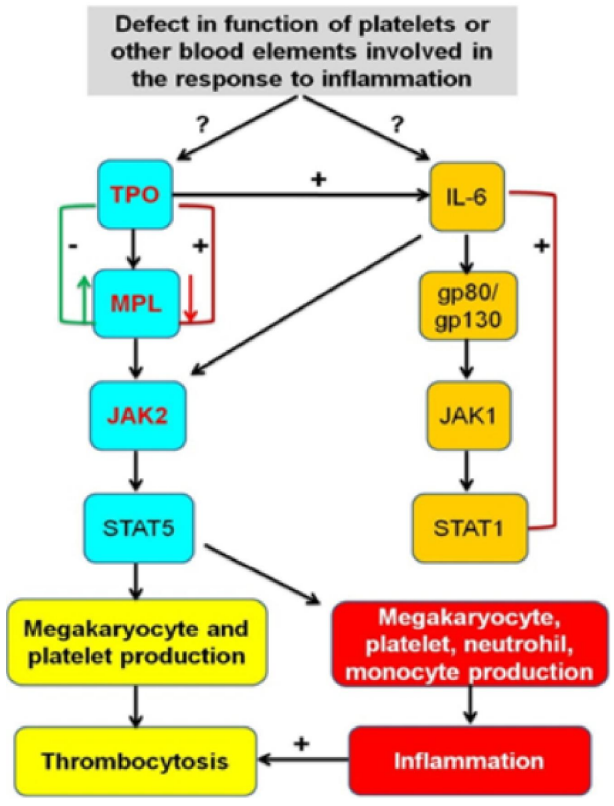

Figure II.9 Links between inflammation and platetet production (simplified)

In conclusion:

The new information from the latest mutations discovered in HT families indicates that we should probably pay more attention to the narrow links between platelet and chronic inflammation

(figure above). Following this reasoning, it is possible that the next new mutations to be discovered in the context of HT concern molecular mechanisms of response to inflammation common to platelets and other myeloid lineages.

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Chapter 3
Clinical presentation

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3.1 Clinical presentation

Chronic thrombocytosis often presents as an incidental finding that is patients being investigated for some other condition and found to have platelet counts above the normal range, thus prompting further blood counts and investigation. Patients are frequently children or young adults, in contrast to patients with myeloproliferative neoplasms (MPNs), who usually present at older ages. However, as hereditary thrombocytosis (HT) is often asymptomatic, HT can also be discovered late in life (see Chapter 6, clinical case 2).

- **Family History**

A family history of thrombocytosis is frequently found, though patients may be the first case in their family. It is also possible that previous familial cases were diagnosed as MPN. The presence of a family history is therefore significant but the absence of a family history does not rule out HT.

- **Bleeding or thrombotic tendency**

It has been established that genetic defects may lead to platelet dysfunction (adhesion, aggregation). Logically certain HT patients may present with a tendency to bleed (menorrhagia, bruises) or, on the contrary, with thrombotic complications (Eyster ME *et al*., 1986; Schlemper RJ *et al*., 1994; Teofili L *et al*., 2010; Giona F *et al*., 2012; personal observations). Some of these events may be severe and/or occur at an unusually young age. Because of the heterogeneous nature of the disorder and the absence of an identified cause for many patients, the frequency of haemorrhagic or thrombotic events in HT is unknown.

- **Myelofibrosis and risk of haematological malignancy**

Studies of HT families with mutations in the *THPO* or *MPL* genes taught us that HT could on occasion evolve in a way similar to MPN, with myelofibrosis developing over time and eventually transforming into acute leukaemia (Posthuma HL *et al*., 2010). In one family, HT was associated with a cutaneous form of malignant lymphoma (Kikuchi M *et al*., 1995). Such cases appear to be rare but as HT is heterogeneous and still poorly understood, caution is advised.

3.2 Mutation-associated clinical presentation

Although the literature on HT is quite limited, it is possible to start classifying HT according to mutations and associated risks. The associated risk(s) as presently reported will need to be confirmed in more patients, and over time.

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– **Mostly benign phenotype**

  – Germline *JAK2* mutations

Currently the literature suggests that HT due to germline *JAK2* mutations is mostly benign. The *THPO* level was low or normal, and reported platelet counts ranged from 437 to 1300 x 10^9/L (Mead AJ *et al*., 2012; Marty C *et al*., 2014; Etheridge SL *et al*., 2014). Affected individuals were typically asymptomatic (Marty C *et al*., 2014; Etheridge SL *et al*., 2014). However in one family, affected members over 40 years of age presented with vascular events (Mead AJ *et al*., 2012). No affected patient had splenomegaly, and no evolution toward myelofibrosis or haematological malignancy was observed.

• **Germline *MPL* K39N SNP and *MPL* P106L mutation** (in the extra-cellular domain)

According to the present literature, the *MPL* K39N SNP 'Mpl Baltimore' and the *MPL* P106L mutation appear to be associated with a benign phenotype, typically isolated thrombocytosis (Moliterno AR *et al*.; 2004, El Harith el-HA *et al*., 2009). There are no reports of evolution toward myelofibrosis or malignant transformation in individuals with *MPL* K39N SNP or P106L mutation. The K39N SNP concerns mostly African Americans, and it was reported that ≈7% of African Americans are heterozygous for *MPL* K39N (Moliterno AR *et al*.; 2004), whereas the P106L mutation concerns mostly Arab populations (El Harith el-HA *et al*., 2009). However the K39N SNP and the P106L mutation may be observed in individuals of different ethnic backgrounds. Logically, individuals homozygous for *MPL* K39N or P106L have much higher platelet counts than heterozygous patients (Moliterno AR *et al*.; 2004).

• **Germline *MPL* W515R mutation**

This mutation has been reported so far in a single family. In this family, affected individuals presented with mild and isolated thrombocytosis, and they were asymptomatic (Vilaine M *et al*., 2012).

– **Possibly more severe phenotype**

  – **Germline *MPL* S505N mutation** (in trans-membrane domain)

A number of HT families, some linked to a common ancestor, have been reported as carrying the activating *MPL* S505N mutation (Ding J *et al*., 2004; Liu K *et al*., 2009). Unfortunately, the initial publications provide little information on the clinical presentation and disease evolution of *MPL* S505N-affected individuals in these families. However, analysis of Italian families showed that carrying the *MPL* S505N mutation seemed to increase the risk of developing splenomegaly, myelofibrosis and various types of thrombotic events (Teofili L *et al*., 2010). No case of transformation to acute leukaemia has been reported. Clinical case 2 in Chapter 6 shows an example of germline *MPL* S505N mutation in an older patient, who developed splenomegaly and myelofibrosis.

• **Germline *THPO* mutations**

HT patients with *THPO* mutations present with markedly elevated serum TPO levels and platelets counts may be mildly elevated (up to 600 x 10^9/L) to over 1500 x 10^9/L (Kondo T *et al*., 1998; Ghilardi N *et al*., 1999; Zhang B *et al*., 2011; Liu K *et al*., 2008). Different clinical phenotypes ranging from asymptomatic thrombocytosis to various types of thrombotic complications have been described in *THPO*-mutated families. Among the reported vascular events, are minor transient ischemic events as well as arterial thrombosis (see Chapter 6, clinical case 1). For instance, Liu *et al*. described brief episodes of fainting and dizziness which responded well to aspirin (Liu K *et al*., 2008). Severe clinical presentations have also been reported, notably limb defects at birth in two families (Graziano C *et al*., 2009; Stockklausner C *et al*., 2012). These observations suggest that chronic TPO overproduction may disturb vasculogenesis. In another family, one member developed a haematological malignancy (multiple myeloma) at a young age (Stockklausner C *et al*., 2012). In addition, myelofibrosis and acute leukaemia have been reported in members of a family with HT due to *THPO* mutation (Posthuma HL *et al*., 2010).

3.3 Summary: clinical issues

A typical patient with HT presents at a young age and may have a positive family history. Symptoms or the family history may include an increased risk of bleeding or, perhaps more frequently, of thrombosis. Progression toward myelofibrosis and acute leukaemia has been reported, particularly in association with germline *THPO* and *MPL* S505N mutations. With the aim of preventing adverse events in individuals and families at risk, HT patients should be followed regularly by a haematologist, and relatives should undergo genetic testing.

Treatment

- **Who**

HT is often an incidental finding. Various forms of HT result from a genetic lesion which causes high platelet counts, either directly (*JAK2* and certain *MPL* activating mutations) or indirectly via an increased production of TPO (*THPO* and certain *MPL* mutations). Ideally, treatment should be adapted to the cause. The present literature, although too limited to allow firm conclusions, suggests that most individuals with germline *JAK2* or *MPL* K39N or P106L mutations are asymptomatic. Moreover, in some cases the increased platelet production may be part of a physiological adaptation to platelet membrane defects. In contrast, patients who carry *THPO* or *MPL* S505N mutations seem to be at risk of thrombotic events and progression toward myelofibrosis or/and leukaemia. In those HT patients at risk of complications, reducing platelet functions and/or platelet counts may be beneficial. Decisions on who to treat therefore need to be based on the context of specific HT patients, their symptoms, their personal context, and their familial history of events.

Treatment modalities

The literature and current understanding of HT are not sufficient to establish clear guidelines for the treatment of HT. The paragraphs below simply aim to provide reasonable therapeutic options or/and advise to consider and to adapt to each HT patient.

- **Reduction of cardiovascular risk factors**

Since certain HT patients seem to be at increased risk of thrombosis due to a germline mutation, it is useful to make every effort to detect and treat other factors that may further increase the risk of thrombosis in the patient. Hence, additional germline mutations associated with thrombophilia should be detected, including mutations in the genes encoding Factor V Leiden and prothrombin, as well as deficiency of anti-thrombin III, protein C, protein S, or fibrinogen. Similarly, cardiovascular disease, elevated cholesterol, diabetes, obesity, or smoking should be detected and treated.

- **Low-dose aspirin**

Low-dose aspirin reduces the incidence of thromboembolic events. Hence it is reasonable to assume that low-dose aspirin may also be useful in preventing or reducing the incidence of thromboembolic events in the context HT. In the absence of guidelines, HT patients may be treated with low-dose aspirin provided that the platelet count is below 1000 x 10^9/L and the patient does not have a contra-indication to aspirin therapy.

- **Anagrelide**

Anagrelide is used in the treatment of essential thrombocythaemia to specifically reduce the production of megakaryocytes and platelets. It is usually prescribed for patients over 60 years of age who present with platelet counts over 1000 x 10^9/L. Similarly, in the absence of guidelines one may consider anagrelide in the context of older HT patients with platelet counts over 1000 x 10^9/L, if there is a need for platelet reduction and/or evidence of a personal or familial risk of thrombosis.

- **Hydroxyurea**

Hydroxycarbamide (hydroxyurea, or HU) is used primarily for the treatment of myeloproliferative neoplasms, where the drug effectively reduces blood counts. In contrast to anagrelide, HU does not act preferentially on megakaryocytes and platelets. Moreover, there is some concern that long term treatment with HU increases the risk of leukaemic transformation. Because of the risk of leukaemia, HU should not be prescribed to young HT patients, or to HT patients with *THPO* mutations, for whom a risk of malignant transformation has been reported. HU may be considered in case of elevation of the leukocyte counts, in addition to the thrombocytosis. However, prior to treatment, it is advised to perform further investigations to explain the change in blood counts, *i.e.* to search for evidence of progression toward a MPN or another form of hematological malignancy.

Follow up

HT patients need long term follow up by a haematologist in order to start or review the need for treatment and to record any events and outcomes. It would be helpful if data and outcomes on such patients could be entered into international databases so as to amass more information on long-term outcomes.

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Chapter 4
Diagnostic algorithm

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Screening for HT should be considered in those individuals with:

– True chronic thrombocytosis (> 450 x 10^9/L platelets and >6 months).

– No identifiable secondary cause of thrombocytosis.

– Young patients.

– Patients with a positive family history.

One should exclude secondary (reactive) causes of thrombocytosis, such as acute or chronic infection (≈50% of thrombocytosis cases are associated with infection), iron deficiency, blood loss, haemolysis, asplenia, malignancies.

Spleen and liver status should be assessed, as asplenia causes thrombocytosis. splenomegaly and/or hepatomegaly are usually absent in HT.

The laboratory work-up includes the following tests:

- Platelet counts, > 450 x 10^9/L. The white blood cell counts and red cell parameters are usually normal.
- Mean platelet volume (MPV) for exclusion of familial microcytic thrombocytosis.
- THPO level, for discrimination of primary/secondary thrombocytosis.
- Negative *JAK2*V617F, *MPL*W515 and *CALR* mutation detection assays.
- Analysis of the *THPO, MPL* and *JAK2* genes.

JAK2 and *MPL* mutations may be present in B and T lymphocytes without necessarily indicating germline distribution. Thus analyses are preferably performed on DNA extracted from non-haematopoeitic tissues. However, for practical purposes, first study blood DNA (easily available, in large quantities) and then confirm the presence of the mutation(s) in DNA obtained from non-haematological tissues (buccal swab, hair follicles, skin biopsy, etc).

The figure below shows the algorithm proposed for the evaluation of suspected HT (Hussein K *et al*., 2014).

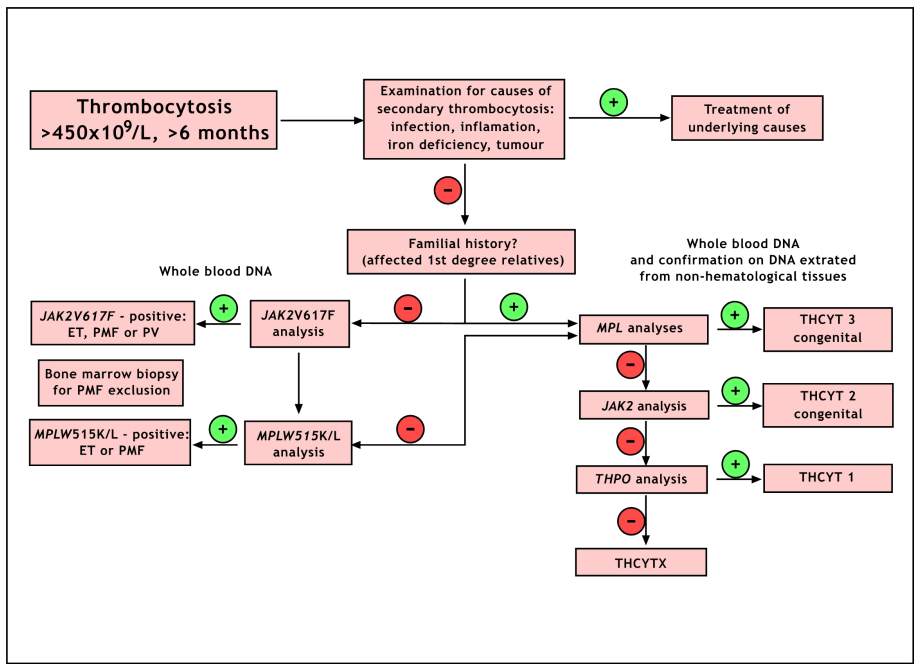

Figure II.10 Algorithm proposed for the evaluation of suspected HT
Abbreviations: THCYT1: thrombocythemia/thrombocytosis type 1 (autosomal dominant); THCYT2, thrombocythemia/thrombocytosis type 2 (autosomal dominant or autosomal recessive); THCYT3, thrombocythemia/thrombocytosis type 3 (autosomal dominant with incomplete penetrance); THCYTX: here, thrombocythemia/thrombocytosis of unknown cause (X= unknown gene).

The diagnosis of HT is certain if a germline mutation is detected in one of the three genes above (such as *JAK2*V617I, *MPL*K39N, *MPL*P106, *MPL*S505N, or *THPO* mutations). Genetic testing may then be suggested to other family members, as a positive result will be associated with an increased risk of having or developing thrombocytosis. Depending on the mutation identified, a benign evolution may be predicted, for instance with germline *JAK2* mutations (Mead AJ *et al*., 2012; Marty C *et al*., 2014) and with certain *MPL* SNP (K39N) or mutation (P106L) (Moliterno AL *et al*., 2004; El-Harith el-HA *et al*., 2009). In contrast, more caution is required in the presence of *THPO* mutations and *MPL* S505N mutations which have been reported as possibly associated with severe complications, including thrombotic events, myelofibrosis and acute leukaemia (Posthuma HL *et al*., 2010; Teofili L *et al*., 2010).

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Chapter 5
Molecular diagnosis – protocols

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This chapter aims to provide practical information to help clinicians and biologists identify HT-causing mutations in patients with chronic thrombocytosis.

5.1 Diagnostic algorithm

When the diagnosis of chronic thrombocytosis is established and a hereditary cause is suspected (see Chapter 4), we suggest the following procedure:

1. Establish whether TPO is increased, or not.
2. In case of high TPO, search first for a germline *THPO* mutation, secondly for a germline *MPL* mutation in the extra-cellular domain.

In case of low or normal TPO, search for a germline mutation in the *MPL* or *JAK2* genes.

3. In the absence of mutation in the *THPO, MPL* and *JAK2* genes, contact a laboratory expert in HT, to perform whole exome sequencing studies.

Since many hospital laboratories do not perform the analyses above, a list of laboratories willing to perform HT studies is provided in the Appendix.

5.2 Molecular diagnosis – protocols

- **Dosage of TPO in blood plasma**

Plasmas are prepared by centrifugation of blood samples at 1400g for 15 min. They are assessed in duplicate or triplicate by enzyme-linked immunosorbent assay (ELISA), using the Quantikine Thrombopoietin (DTP00B) kit (R & D Systems Inc., Abingdon, UK) according to the manufacturer's instructions. Values (average of duplicates or triplicates) are expressed as pg/mL.

- **Detection of HT-causing mutations**

Origin of DNA samples

The different assays designed for the detection of germline mutations causing HT are often used on genomic DNA extracted either from whole blood or from blood cells. However, to affirm the germline nature of these mutations, it is necessary to confirm the presence of the mutation identified in blood in non- haematopoietic tissues, generally in DNA extracted from buccal swabs, hair follicles, or a skin biopsy.

Conventional sequence analysis of the *THPO, MPL* and *JAK2* genes

Exons 1-6 of *THPO*, exons 1-12 of *MPL* and exons 12-15 of *JAK2* are amplified by polymerase chain reaction (PCR) and subjected to direct sequencing. The primers to be used were previously published (Ding J *et al*., 2004; Liu K *et al*., 2008; Vilaine M *et al*., 2011) except for *MPL* exons 9 and 10:

- Forward exon 9: 5'-GGAATCTCCGACCGCCTGG-3',
- Reverse exon 9: 5'- GCTGTGCGGCTTTGGTGC-3',
- Forward exon 10: 5'-TATGGGCCGAAGTCTGACCCTTT-3',
- Reverse exon 10: 5'-CAGAGCGAACCAAGAATGCCTGT-3'.

Twenty-five ng of total genomic DNA are used in a 50 μl PCR mix containing 10 μM of corresponding primers. PCR products were analyzed on 1% agarose gel and then sequenced by Sanger method or BigDye ®Terminator

High resolution melting (HRM) curve analysis of *MPL* exon 10

This sensitive method is recommended to screen DNA samples in series of patients for whom a mutation in *MPL* exon 10 is suspected. Once a mutation is detected, sequencing is necessary to confirm the mutation.

Ten ng of total genomic DNA for detection threshold tests, are amplified with the Probes Master kit (Roche Applied Sciences, Mannheim, Germany), 3.2 mM MgCl2, Resolight fluorescent dye (Roche Applied Sciences, Mannheim, Germany), 0.4 microM of primers 5'-AGCCTGGATCTCC TTGGTGA-3' and 5'-GCGGTACCTGTAGTGTGCAG-3' in a 10 μL final volume (HRM PCR). Amplification and melting conditions are as follows: 95°C for 10 min, 50 cycles at 95°C for 10

seconds, 58 to 65°C for 20 seconds touch-down annealing programs (0.5°C a step) and 72°C for 20 seconds elongation, and a melt from 65 to 95°C at 0.01°C per second. Fifty fluorescence acquisitions per °C are done, performed on a LC480 apparatus (Roche Applied Sciences, Mannheim, Germany). HRM curve analysis is performed using normalized, temperature shifted difference plot. Tm (melting temperature) is also used to confirm results obtained with the previous analysis (mutations give a left -low temperature- sided shoulder on the melting peak graph). Negative controls are used as reference curves to generate the difference plot. Genomic DNA from patients harboring *MPL* W515L/K mutations are used as positive controls.

A similar HRM-based approach to the detection of *MPL* exon 10 mutations can be adapted to cDNA (Schnittger S *et al*., 2009).

Next Generation Sequencing of the *THPO, MPL, JAK2* and *GSN* genes

Currently we are not aware of any diagnostic laboratory which uses the next-generation sequencing (NGS) technology solely for the diagnosis of HT-causing mutations. Two main reasons are the small number of genes currently associated with HT, and their very recent discovery (*JAK2, GSN*). Thus these four genes are still analyzed one by one.

Whole exome sequencing

The recent discovery of the *GSN* mutation in a HT family showed that "open" techniques are necessary to identify new causes of HT in families with no mutation in the *THPO, MPL, JAK2* or *GSN* genes. Such studies are now performed mostly using whole exome sequencing technologies, in research laboratories interested in searching for new HT-causing mutations (a list of laboratories is provided in the Appendix).

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Chapter 6
Clinical cases – Presentation, diagnosis and follow-up

Eric Lippert, Serge Carillo, Mathias Vilaine, Thierry Lavabre-Bertrand, Sylvie Hermouet

Case 1 – Germline *THPO* mutation – Isolated thrombocytosis

Eric Lippert
University Hospital Bordeaux, France

Clinical Presentation	A 13 year old boy with high platelet count (750 x 10^9/L) No symptoms
Patient and family History	The mother and the mother's father have high platelets (600-750 x 10^9/L). The latter also witnessed an iliac artery thrombosis. No other thrombotic complication in the family. A male brother has normal platelet counts.
Examination Findings	No abnormalities were identified on examination. Ultrasound of the abdomen did not reveal any abnormality.

Laboratory parameters

Hb (g/L)	Hct (%)	RBC (x10^{12}/L)	MCV (fL)	WBC (× 10^9/L)	Pts (× 10^9/L)	Bone marrow	TPO (pg/mL)	*JAK2* V617F	*MPL exon10*	CALR
13.2	39	4.86N	80.5	7.0	750	Not done	106	NEG	NEG	NEG

TPO was above the normal range in all affected family members (mother: 200 pg/mL, grandfather: 218 pg/mL)

Molecular diagnosis	Sequencing of all exons of the *THPO* gene revealed a mutation which affects the splicing site of exon 3: NM_000460:c.141G>A
Clinical follow-up and events	None reported in the index case or his mother. The grand father has had iliac artery thrombosis at the age of 65. At age 73, he suffered severe chronic obliterative arteriopathy of the inferior limbs, leading to amputation and severe leg ulcers. He is also affected by ischemic cardiopathy.

Abbreviation. NEG: negative

Case 2 – Germline *MPL* S505N mutation – Thrombocytosis with complications

Serge Carillo, Thierry Lavabre-Bertrand
University Hospital of Nîmes, France.

Clinical Presentation

A 75 year-old man with slightly elevated platelet count (500 x 10^9/L)
No symptoms

Patient and family History

Unknown, not married, no children

Examination Findings

No abnormality was identified on examination.
Ultrasound of the abdomen did not reveal any abnormality.

Laboratory parameters

Hb (g/L)	Hct (%)	RBC (x10^{12}/L)	MCV (fL)	WBC (× 10^9/L)	Pts (× 10^9/L)	Bone marrow	TPO (pg/mL)	*JAK2* V617F	*MPL exon10*	CALR
Nl	Nl	Nl	Nl	Nl	500	"ET"	NA	NEG	S505N	NEG

Bone marrow smear analysis revealed an elevated number of giant megakaryocytes with hyperlobulated nuclei ("staghorn nuclei") similar to what can be observed in the context of essential thrombocythemia (ET)

Molecular diagnosis

High resolution melting (HRM) curve analysis plus Sanger sequencing on both granulocyte and lymphocyte genomic DNA of *MPL* exon10: NM_005373:c.1513G>C.
Mutant allelic burden: 50% in both type of cells (granulocytes, lymphocytes)

Clinical follow-up and events

Evolution toward myelofibrosis with peripheral erythroblastosis, dacryocytosis, and bone marrow histology typical of myelofibrosis.
Low red cell mass (RCM: -46%) associated with a slightly elevated plasmatic volume (+ 16%), resulting in lowered haemoglobin concentration.

Abbreviations. Nl: normal; NA: not available; NEG: negative

Case 3 – Germline *MPL* W515R mutation – Isolated thrombocytosis*

Mathias Vilaine, Sylvie Hermouet*
Institution: University Hospital of Nantes, France.

Clinical Presentation	A 36 year old woman with slightly elevated platelet count (660 x 10^9/L). No symptoms
Patient and family History	Father with chronic thrombocytosis Grand-mother (deceased) reported with chronic thrombocytosis
Examination Findings	No abnormality was identified on examination Ultrasound of the abdomen did not reveal any abnormality No personal or familial history of thrombosis, bleeding, or myeloproliferative neoplasm

Laboratory parameters

Hb (g/L)	Hct (%)	RBC (x10^{12}/L)	MCV (fL)	WBC (× 10^9/L)	Pts (× 10^9/L)	Bone marrow	TPO (pg/mL)	*JAK2* V617F	MPL *exon10*
Nl	Nl	NA	Nl	Nl	660	Nl	Not detectable	NEG	W515R

Molecular diagnosis	High resolution melting (HRM) curve analysis and Sanger sequencing of *MPL* exon 10 in genomic DNA extracted from granulocytes, T- lymphocytes, buccal cells and hair follicles, from the father and daughter: all cell types carried the same mutation NM_005373:c.1543T>C (MPL W515R) in a heterozygous manner.
Clinical follow-up and events	No complication for the father and the patient

Abbreviations. Nl: normal; NA: not available; NEG: negative.

(*) Vilaine M, Gourain V, Cleyrat C, et al. Germline *MPL*W515R mutation in a family with isolated thrombocytosis. ASH Annual Meeting Abstr. 2012;120 :1764.

Appendix

Labs performing molecular diagnosis

The following laboratories offer sequencing for the following HT-associated genes.

Genes	Country	Name	Institution	E-mail address
THPO	France	Dr. Eric Lippert	CHU Bordeaux	eric.lippert@chu-bordeaux.fr
MPL	France	Dr. Serge Carillo	CHU Nîmes	sergecarillo@yahoo.fr
THPO *MPL* *JAK2*	Germany	Dr. Susanne Schnittger	Munich Leukemia Laboratory (MLL)	susanne.schnittger@mll-online.com
THPO *MPL* *JAK2*	Spain	Dr. Beatriz Bellosillo Paricio	Hospital del Mar Barcelona	BBellosillo@parcdesalutmar.cat
THPO *MPL* *JAK2*	The Czech Republlic	Dr. Jiri Schwarz	Hematology and Blood Transfusion Institute, Prague	Jiri.Schwarz@uhkt.cz
Exome sequencing (searching for new candidate genes causing HT)	France	Dr. Sylvie Hermouet Dr. Mathias Vilaine	Inserm UMR 892 Université de Nantes	sylvie.hermouet@univ-nantes.fr mathias.vilaine@univ-nantes.fr

The following laboratories offer sequencing for the following CE-associated genes.

Institution	Contact	Genetic Testing For						
		HBB	HBA	BPGM	EPOR	VHL	PHD2 (EGLN1	HIF2a (EPAS1)
Hematology Department, Centro Hospitalar e Universitário de Coimbra; PORTUGAL	Celeste Bento celeste.bento@chc.min-saude.pt	All exons	All exons	All exons	Exon 7-8	All exons	All exons	Exon 9,12
Institute for Clinical Transfusion Medicine and Immunogenetics (IKT Ulm) and Dept. of Pediatrics and Adolescent Medicine (Ped) ; GERMANY	Klaus Schwarz (IKT); Holger Cario (Ped) (klaus.schwarz@uni-ulm.de; holger.cario@uniklinik-ulm.de)	All exons	All exons	No	Exon 7-8	All exons	All exons	Exon 9-16
Belfast City Hospital; UK	Melanie Percy; Mary Frances McMullin (melanie.percy@belfasttrust.hscni.net)	All exons	All exons	No	Exon 7-8	All exons	Exon 1-4	Exon 12
MLL - Munich Leukemia Laboratory; GERMANY	Susanne Schnittger (Susanne.schnittger@mll-online.com)	No	No	No	Exon 8	All exons	P 317 and R371 only	Exon 12
Inselspital Bern; SWITZERLAND	Naomi Porret; Elisabeth Oppliger (naomi.porret@insel.ch; elisabeth.oppliger@insel.ch)	No	No	No	Exon 7-8	All exons	No	No
Hospital del Mar, Barcelona; SPAIN	Beatriz Bellosillo (94161@imas.imim.es)	No	No	No	Exon 8	All exons	P 317 R371 and flanking regions	M535, G537 and flanking regions
Department of Woman, Child and General and Specialist Surgery, Second University of Naples; ITALY	Silverio Perrotta (silverio.perrotta@unina2.it)	All exons	All exons	No	Exon 7-8	All exons	All exons	Exon 9,12
University Medical Center Utrecht; NETHERLANDS	Richard van Wijk (r.vanwijk@umcutrecht.nl)	All exons	All exons	All exons	Exon 7-8	All exons	All exons	Exon 9,12
Dept of Medicine DIMED, Internal Medicine, University of Padua; ITALY	Maria Luigia Randi (marialuigia.randi@unipd.it)	No	No	No	Exon 8	All exons	Exon 1,2,3	Exon 12
Center for Human Genetics, Molecular Haemato-oncology Unit, CHU Sart-Tilman, Liege; BELGIUM	Frédéric Lambert (flambert@chu.ulg.ac.be)	No	No	No	Exon 7-8	All exons	All exons	Exon 12
Hematology Laboratory, Brest University Hospital; FRANCE	Valerie Ugo (valerie.ugo@chu-brest.fr)	No	No	No	Exon 7-8	No	No	No
Laboratoire de Génétique Moléculaire, Hôpital Européen Georges Pompidou, Paris; FRANCE	Anne-Paule Gimenez-Roqueplo (anne-paule.gimenez-roqueplo@egp.aphp.fr)	No	No	No	No	All exons	All exons	Exon 12
Laboratoire de Génétique moléculaire, CHU Hotel-Dieu; Nantes; FRANCE	Stéphane Bézieau (francois.girodon@chu-dijon.fr; betty.gardie@inserm.fr; sylvie.hermouet@univ-nantes.fr)	No	No	No	Exon 7-8	All exons	All exons	Exon 12
Dept. of Biology (Bio), Dept. of Pediatrics (Ped), Faculty of Medicine and Dentistry, Palacky University and University Hospital; CZECH REPUBLIC	Vladimir Divoky (Bio); Dagmar Pospisilova (Ped) (vladimir.divoky@upol.cz; dagmar.pospisilova@fnol.cz)	All exons	All exons	Not done	Exon 7-8	All exons	All exons	Exon 9,12
Instutite of Hematology and Blood Transfusion; CZECH REPUBLIC	Jirí Schwarz; Jana Marková (jiri.schwarz@uhkt.cz; jana.markova@uhkt.cz	Not done	Not done	Not done	Exon 7-8	All exons	Exon 2-4	Exon 12
Karolinska University Hospital, Stockholm; SWEDEN	Britta Landin; Soheir Beshara (britta.landin@karolinska.se; soheir.beshara@karolinska.se)	All exons	All exons	Exons 1,3,4	Exon 8	All exons	Not done	Not done