OTHER

Review

Erythrocytosis: genes and pathways involved in disease development

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Erythrocytosis is a blood disorder characterised by an increased red blood cell mass. The most common causes of erythrocytosis are acquired and caused by diseases and conditions that are accompanied by hypoxaemia or overproduction of erythropoietin. More rarely, erythrocytosis has a known genetic background, such as for polycythaemia vera and familial erythrocytosis. The majority of cases of polycythaemia vera are associated with acquired variants in *JAK2*, while familial erythrocytosis is a group of congenital disorders. Familial erythrocytosis type 1 is associated with hypersensitivity to erythropoietin (variants in *EPOR*), types 2-5 with defects in oxygen-sensing pathways (variants in *VHL, EGLN1, EPAS1, EPO*), and types 6-8 with an increased affinity of haemoglobin for oxygen (variants in *HBB, HBA1, HBA2, BPGM*). Due to a heterogenic genetic background, the causes of disease are not fully discovered and in more than 70% of patients the condition remains labelled idiopathic.

The transfer of next-generation sequencing into clinical practice is becoming a reality enabling detection of various variants in a single rapid test. In this review, we describe the current research on erythrocytosis gene variants and the mechanisms associated with disease development, along with the currently used diagnostic tests.

Keywords: *erythropoiesis, erythrocytosis, polycythaemia, genetic disease, genetic testing.*

INTRODUCTION

The process of erythropoiesis in the human body generates 2.4 million red blood cells (RBC) every second[1,2]. Haematopoietic stem cells differentiate from nucleated erythroid progenitors into mature non-nucleated erythrocytes[2]. Erythropoiesis is tightly controlled by erythropoietin (EPO) and signalling through its receptor, EPOR[3]. Defects in erythropoiesis can lead to erythrocytosis, a disorder that is characterised by elevated red cell mass (RCM)[3-7].

Erythrocytosis is suspected when an abnormally high haematocrit, haemoglobin concentration, or RBC count are detected. These parameters depend on plasma volume, as reduced plasma volume leads to relative (false) erythrocytosis. Absolute (true) erythrocytosis is defined by a RCM >125% of predicted for age and sex. RCM measurement

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is rarely possible nowadays. It has been shown that a male/female with a haematocrit of ≥0.60/0.56 will always have absolute erythrocytosis[7]. According to the new 2016 World Health Organisation (WHO) criteria, erythrocytosis is diagnosed with lower thresholds for haemoglobin concentration and haematocrit (>165 g/L or >0.49 in males and >160 g/L or >0.48 in females)[8]. In some cases a haematocrit threshold of >0.52/>0.48 in males/females is still used[9].

Erythrocytosis can be classified as congenital or acquired, and the origins of both can be primary (i.e., an intrinsic defect in RBC) or secondary (i.e., extrinsic to RBC)[6,7,10,11]. The most common causes of erythrocytosis are acquired, and they develop due to diseases and conditions that are accompanied by hypoxaemia or overproduction of EPO. Conditions that can lead to central hypoxic processes include smoking, sleep apnoea, carbon monoxide poisoning, and a high-altitude habitat[6]. Several heart, lung, and kidney diseases can also result in erythrocytosis[6]. Pathological EPO production (i.e., secondary acquired erythrocytosis) is observed in several cancers, such as cerebellar haemangioblastoma, meningioma, parathyroid carcinoma, and some other types of carcinoma[3,6]. Erythrocytosis following allogeneic haematopoietic stem cell transplant and renal transplant has also been reported[7,12,13]. Erythrocytosis is a common side effect of drug consumption, as with diuretics, recombinant human EPO, and testosterone replacement therapy[14]. Chronic diuretic use can lead to a decrease in the plasma volume and relative erythrocytosis. The use of androgens elevates endogenous EPO production and consequently haemoglobin concentration by a mechanism similar to that of recombinant EPO administration.

Less often, erythrocytosis has a genetic background, acquired in polycythaemia vera (PV), the most common myeloproliferative neoplasm, and congenital or familial erythrocytosis (ECYT), a rare genetic disorder. Acquired variants in the Janus tyrosine kinase 2 gene (*JAK2*) are indicative of PV[15]. Congenital ECYT has a heterogeneous genetic background and it has been divided into eight types, based on the genes affected (**Table I**). Primary erythrocytoses, PV and ECYT1, are the consequences of constant activation of the EPO-EPOR signalling pathway due to variants in the *JAK2* or *EPOR* genes, with the levels of serum EPO usually being below normal[3]. Secondary congenital erythrocytoses (i.e., ECYT2-8) develop due to a mechanism extrinsic of the erythroid cells, and here the levels of serum EPO are usually normal or elevated[3]. The mechanism underlying ECYT2-5 is an altered hypoxia inducible factor (HIF)-EPO oxygen-sensing pathway which is due to inherited variants in the *VHL, EGLN1, EPAS1,* or *EPO* genes[16]. Finally, the mechanism that leads to ECYT6-8 is an increased affinity of haemoglobin for oxygen, due to variants in the *HBB, HBA1, HBA2,* and/or *BPGM* genes[6].

The incidence of PV is 0.4 to 2.6 cases per 100,000 inhabitants, while the prevalence in Europe is undetermined[17]. The incidence of PV in the USA (more precisely, in Olmsted County, Minnesota) from 1935 to 1989 was estimated to be 1.9 cases per 100,000 inhabitants (males, 2.8 and females 1.3 per 100,000 inhabitants per year). The incidence in Japan is lower than in the USA or Europe. Both the incidence and prevalence of ECYT are low, but precise epidemiological data are unavailable because of the limited disease classification and incomplete genetic diagnosis.

Table I - *Genes associated with erythrocytosis*

Gene	Effect on protein	Type
JAK2	Gain of function, impaired auto-inhibitory domain	PV
EPOR	Gain of function, increased activation of signalling cascade	ECYT1
VHL	Loss of function, impaired degradation of HIF2α	ECYT2
EGLN1 (PHD2)	Loss of function, impaired degradation of HIF2α	ECYT3
EPAS1 (HIF2A)	Gain of function, increased stability in normoxia	ECYT4
EPO	Gain of function, increased expression in normoxia	ECYT5
HBB	Loss of function, increases Hb affinity for O2	ECYT6
HBA1 & HBA2	Loss of function, increases Hb affinity for O2	ECYT7
BPGM	Loss of function, impaired synthesis of 2,3-BPG	ECYT8

PV: polycythaemia vera; ECYT: familial erythroctyosis; HIF-2α: hypoxia-inducible factor 2-alpha; Hb: haemoglobin; O2: oxygen; 2,3-BPG: 2,3-bisphosphoglycerate.

Recently, knowledge about erythrocytosis has evolved relatively rapidly, as new genes involved in the development

of ECYT have been described. Erythrocytosis detection tests have been updated regularly, including customised next-generation sequencing (*NGS*) gene panels[18].

In this review, we initially describe the process of erythropoiesis and its regulation and review the gene variants associated with erythrocytosis. An up-to-date review of the HIF-EPO pathway was published in 2019 by Lappin and Lee[19]. In this paper, we focus on additional pathways that are associated with erythrocytosis and review the genetic testing in current clinical practice. A new overview on erythrocytosis will bring new insights into disease development and diagnostics.

Erythropoiesis and its regulation

Erythropoiesis is defined as the proliferation, differentiation, and maturation of haematopoietic stem cells into fully functional mature somatic RBC[20-26] (**Figure 1**). This process is directed by the cell cytokines and the signalling pathways that are directly or indirectly involved in erythrocyte maturation and oxygen sensing[27,28]. The main, important cytokines are the granulocyte and macrophage colony-stimulating factors (i.e., GM-CSF, M-CSF), the interleukins (e.g., IL-3), and EPO, the concentrations of which are tightly regulated inside the bone marrow.

Erythroid differentiation is divided into engagement and maturation phases. In the engagement phase, multipotent haematopoietic stem cells differentiate into unipotent erythroid progenitor proerythroblasts. In the maturation phase, these proerythroblasts further differentiate into erythroblasts (during a ribosome synthesis phase), normoblasts (during a haemoglobin accumulation phase), and reticulocytes (during the phase of ejection of the nucleus). Reticulocytes are released from the bone marrow into the bloodstream, where it takes them up to 2 days to develop into mature erythrocytes[20,21,25].

Autophagy has an important role in the correct formation of erythrocytes, as it participates in the clearance of "unnecessary" cellular organelles, such as the nucleus, ribosomes, and mitochondria[29]. As erythrocytes do not have a nucleus, genes, and gene variants that might affect erythropoiesis can only be expressed up to the normoblast phase. Haemoglobin and other proteins remain active until programmed cell death kicks in, which is similar to apoptosis, and is known as eryptosis[30].

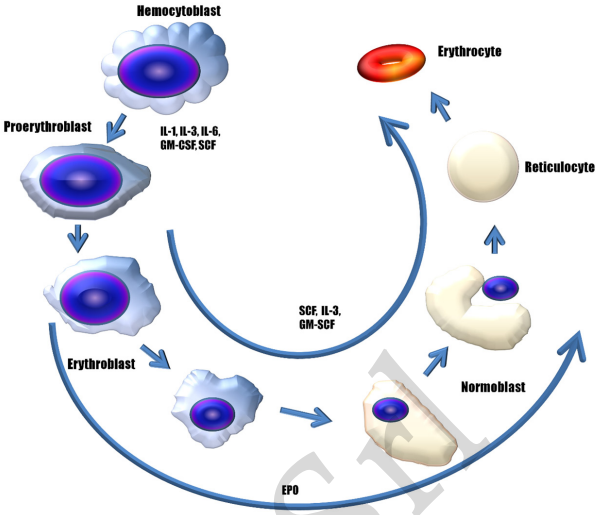

Figure 1 - Role of erythropoietin and other cytokines in erythropoiesis

Scheme of the maturation and differentiation of stem cells to fully developed erythrocytes. The cytokines interleukin (IL)-1, IL-3, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and stem-cell factor (SCF) regulate the development of haematopoietic stem cells into proerythroblasts. Cytokines SCF, IL-3, GM-SCF, and erythropoietin (EPO) further evolve the proerythroblasts to erythrocytes. EPO influences the differentiation of cells expressing EPO receptor (EPOR) on the cell surface, up to the formation of reticulocytes. Erythroblasts evolve into normoblasts, which lose the nucleus and develop into reticulocytes, and later into erythrocytes.

MECHANISMS LEADING TO ERYTHROCYTOSIS

The hypoxia-inducible factor – erythropoietin pathway

The prime regulator of erythropoiesis is EPO, which is a protein hormone that is produced mainly by the kidney[19]. The HIF transcription factor family regulates EPO expression. In humans there are three alpha subunit paralogues: HIF1A, EPAS1 (HIF2A), and HIF3A. All three HIFA paralogues form a transcription complex with HIF beta subunit, aryl hydrocarbon receptor nuclear translocator (ARNT, also known as HIF1B). ARNT is constitutively expressed, while HIFA are regulated through sensing of the oxygen concentrations in the cell cytoplasm[31,32]. The main regulator of EPO production in the kidneys is endothelial Per-Arnt-Sim (PAS)-domain-containing protein 1 (EPAS1, also known as HIF2A)[33]. Two additional proteins are crucial for this HIF–EPO pathway regulation: the Egl nine homolog 1 (EGLN1, also known as prolyl hydroxylase domain-containing protein 2; PHD2); and von Hippel-Lindau disease tumour suppressor (VHL)[34-36] (**Figure 2A**).

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Under normal oxygen conditions, HIFA is hydroxylated by EGLN1, with this hydroxylation being recognised by VHL, which leads to HIFA ubiquitination and degradation in the proteasome[16,32,37]. Under low oxygen concentrations, EGLN1 is not active, and HIFA is translocated into the nucleus, where it forms a transcription complex with ARNT and CREBBP/p300 HAT; this results in the transcription of numerous hypoxia-inducible genes, including *EPO*, which influence the growth of erythroid progenitor cells[38]. Once EPO is released into the bloodstream and transported to the bone marrow, it binds to EPOR on the surface of erythroblasts, thus leading to activation of the EPO–EPOR signalling pathway[39].

The erythropoietin – erythropoietin receptor pathway

The EPO ligand can bind to two EPOR, which results in receptor homodimerisation and conformational change. The EPOR cytokine receptor does not have any kinase activity, but it is associated intracellularly with JAK2, which is crucial for receptor activation via phosphorylation. The conformational change in the EPOR dimer brings two JAK2 molecules into proximity, which leads to their autophosphorylation and transphosphorylation, as well as to phosphorylation of tyrosine residues in the EPOR cytoplasmic region. This results in activation of the downstream signalling molecules, which include the JAK2/STAT5, MAPK/ERK, PI3K/AKT, and protein kinase C pathways[39]. The JAK2 self-activation is prevented by the pseudokinase autoinhibitory domain on one of the JAK2 molecules, which blocks the kinase domain of the other JAK2. The EPO-EPOR signal is terminated via the EPOR intracellular inhibitory domain PTPN6/SOCS-3. The SH2B adapter protein 3 (SH2B3, also known as lymphocyte-specific adapter protein; LNK) works as a negative feedback mechanism for EPOR signal termination[40-42] (**Figure 2B**).

Activation of the EPO-EPOR signalling pathway in erythroblasts results in the transcription of various genes that are involved in cell proliferation, differentiation, prevention of apoptosis, and iron regulation, leading to RBC maturation[42].

Regulation of haemoglobin – oxygen affinity

The haemoglobin within mature RBC carries oxygen from the lungs to the peripheral tissues. Haemoglobin has a tetrahedral structure made up of four haem groups and four globin groups. Oxygen is bound to the iron atom within each haem group, enabling one haemoglobin to carry up to four oxygen atoms. In humans, several haemoglobin subunits exist: HBA1, HBA2, HBB, HBD, HBG1, and HBG2. The fully functional haemoglobin in adults, HbA, comprises two HBA and two HBB subunits. The *HBA1* and *HBA2* genes are paralogues, with an almost identical DNA sequence[43]. *HBG1* and *HBG2* are expressed only during foetal development, when two HBG together with two HBA subunits constitute foetal haemoglobin (HbF). HbF is normally replaced by adult haemoglobin (HbA) at birth. HBD integrates into only 3% of adult haemoglobin.

The enzyme bisphosphoglycerate mutase (BPGM) transforms 1,3-bisphosphoglycerate (BPG) into 2,3-BPG, which pushes the chemical equilibrium into the release of oxygen from the haemoglobin[44] (**Figure 2C**). A haemoglobin variant with a higher affinity for oxygen or lower BPGM activity can decrease the oxygen release from haemoglobin, which will lower the free oxygen in the blood, resulting in tissue hypoxia.

GENE VARIANTS ASSOCIATED WITH ERYTHROCYTOSIS

Erythrocytosis is associated with several gene variants that have been reported in patients with symptoms associated with erythrocytosis (i.e., increased haemoglobin concentration, haematocrit, RBC, RCM). The Online Mendelian Inheritance in Man (OMIM) database[45] specifies that two genes are involved in somatic erythrocytosis or PV; *JAK2*[46] and *SH2B3*[38], and nine genes are involved in familial erythrocytoses *ECYT*1-8; *EPOR*[47-49]; *VHL*[50-52]; *EGLN1*[53-55]; *EPAS1*[56,57]; *EPO*[58,59]; *HBB*[4,60]; *HBA1*[43,60]; *HBA2*[43,60]; and *BPGM*[44,60] (**Table I**). Each of these genes is involved in a key regulatory mechanism of erythropoiesis including the HIF-EPO pathway in kidneys, the EPO-EPOR signalling pathway in the bone marrow, and the regulation of haemoglobin-oxygen affinity in RBC (**Figure 2**). Several other genes have been identified in patients with erythrocytosis, but their role in the development of erythrocytosis was not confirmed[10].

The Leiden Open Variation Database (LOVD) is an open-source DNA variation database system that is designed to collect and display all variants of any specific gene[61]. Common variations are often non-pathogenic, while pathogenic variants are frequently present in populations at low proportions. Several hundred variants

of several genes have been deposited in LOVD and have been associated with PV and ECYT. Although LOVD is regularly updated, not all identified variants are yet deposited in the database. In **Table II** we have listed 58 variants of the *HBB, EPAS1, VHL, EGLN1, EPOR, EPO,* and *BPGM* genes that have not yet been deposited in LOVD, which were extracted from the literature in the PubMed database.

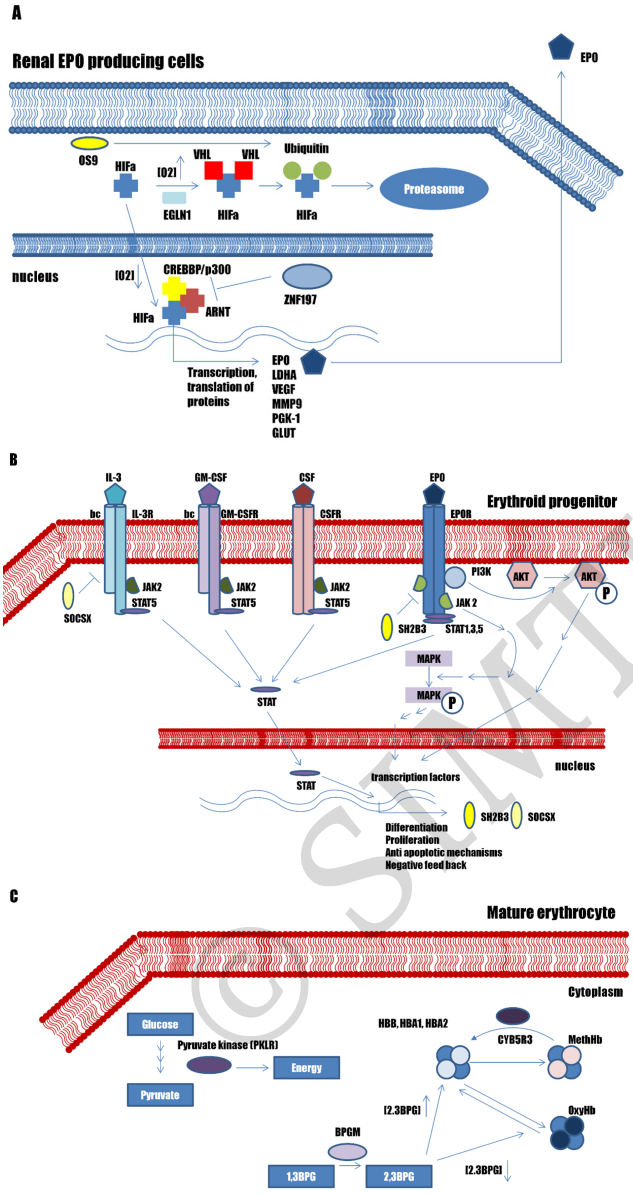

Figure 2 - Pathways associated with erythrocytosis
(A) The genes involved in the hypoxia-inducible factor (HIF) – erythropoietin (EPO) pathway in the kidneys. (B) Genes involved in the EPO – erythropoietin receptor (EPOR) signalling pathway in erythroid progenitors. (C) Genes affecting haemoglobin-oxygen affinity in erythrocytes.

JAK2 activation in polycythaemia vera

PV is a clonal haematopoietic stem-cell disorder that is characterised by increased total blood volume and proliferation of erythroid, granulocytic, and megakaryocytic elements of the bone marrow[41]. The somatic *JAK2* V617F exon 14 variant is the cause of the disorder in 95% of affected patients, while 3% have variants in *JAK2* exon 12[41,62-64]. These two exon site variants appear to have similar activating effects. The *JAK2* V617F variant triggers JAK2 signalling, which leads to increased sensitivity to cytokines (including EPO) and cytokine-independent cell proliferation and survival.

The disease mechanism is based on inactivation of the JAK2 pseudokinase autoinhibitory domain, which is responsible for inhibition of JAK2 self-activation[41]. Two variants in exon 13 have been associated with erythrocytosis[65,66], and heterozygous variants in *JAK2* and *EGLN1* with familial erythrocytosis[67]. Variants of the *JAK2* gene may be present in several other diseases, such as essential thrombocythaemia and primary myelofibrosis, although the symptoms of affected patients can be very different[41,68].

Specific mutations of the calreticulin (*CALR*) gene and thrombopoietin receptor (*MPL*) gene are very rare in PV. However, more than 50% of PV patients were found to have at least one mutation other than well-described driver mutations, with *TET2* and *ASXL1* being the most commonly involved genes[41,68]. *SH2B3* variants, germline or somatic, are not frequently associated with erythrocytosis but may cause primary erythrocytosis similar to ECYT1[40,69,70].

Erythropoietin receptor activation in familial erythrocytosis type 1

Familial erythrocytosis type 1 (ECYT1) is an autosomal dominant genetic disorder that is associated with more than 28 germline *EPOR* variants[59,71-78]. Different frameshift variants in exon 8 have been identified in families in which several members show symptoms of the disease. The mechanism underlying the frameshift variants is a truncation of the EPOR intracellular C-terminal region, which is responsible for negative feedback regulation of the receptor[79,80]. These variants enable EPO-EPOR signal transduction even at low serum EPO levels, and they prevent feedback inhibition through the SH2B3 and PTPN6/SOCS-3 inhibitory domain[47,50].

In 2018, Pasquier *et al.* reported an additional mechanism

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Table II - *Genetic variants associated with congenital erythrocytosis*

Gene	Location	DNA change (published as)	Protein change	dbSNP ID	Number reported	Genotype	PMID (reference)	Remarks
EPOR	**Exon 8**	NM_000121.4: c.1161_1186del	p.(Pro388Hisfs*3)	/	1/1,192	Heterozygous	29790589	
		NM_000121.4: c.1166dup	p.(Gly390Trpfs*10)	/	1/1,192	Heterozygous	29790589	
		NM_000121.4: c.1202C>G	p.(Ser401*)	/	1/1,192	Heterozygous	29790589	
		NM_000121.4: c.1307T>A	p.(Leu436*)	/	1/1,192	Heterozygous	29790589	
		NM_000121.4: c.1310G>A	p.(Arg437His)	rs62638744	1/58		26010769	
		NM_000121.4: c.1362C>A	p.(Tyr454*)	/	1/1,192	Heterozygous	29790589	
	Other genetic variants are listed in Vočanec *et al.* (2019)[59]							
VHL	**Exon 1**	NM_000551.3: c.28G>T	p.(Glu10*)	/	1/163	Heterozygous	24115288 (4)	
		NM_000551.3: c.154G>T	p.(Glu52*)	rs373068386	1/125	Heterozygous	27651169 (10)	
		NM_000551.3: c.235C>T	p.(Arg79Cys)	rs200885420	1	Compound heterozygous	15642680	Together with variant p.(Leu188Val)
		NM_000551.3: c.241C>G	p.(Pro81Ala)	/	1/163	Compound heterozygous	24115288 (4)	Together with variant p.(Gly144Arg)
		NM_000551.3: c.290C>T	p.(Pro97Leu)	/	1/1,192	Heterozygous	29790589	
		NM_000551.3: c.311G>T	p.(Gly104Val)	rs869025630	1/43	Heterozygous	15642664	
	Intron 1 (cryptic exon E1')	NM_000551.3: c.340+574A>T	/	rs982745672	1	Compound heterozygous	29891534 (51)	Together with variant p.(Gln164His)
		NM_000551.3: c.340+694_711dup	p.(Trp159*) (impact on potential X1 protein)	/	2	Compound heterozygous	29891534 (51)	Together with variant p.(Arg200Trp) or p.(Gly144Arg)
		NM_000551.3: c.340+770T>C	p.(Ser179Pro) (impact on potential X1 protein)	rs1346312258	4	Compound heterozygous	29891534 (51)	Together with variant p.(Arg200Trp) or p.(Asp143Asp)
		NM_000551.3: c.340+816A>C	p.(*194Serext*24) (impact on potential X1 protein)	rs1031288121	3 (family members)	Homozygous / heterozygous	29891534 (51)	
	Exon 2	NM_000551.3: c.370A>G	p.(Thr124Ala)	/	2 (family members)	Compound heterozygous	23772956	Together with variant p.(Leu188Val)
		NM_000551.3: c.376G>T	p.(Asp126Tyr)	rs104893831	2 (family members)	Heterozygous	12393546	
		NM_000551.3: c.413C>T	p.(Pro138Leu)	rs780178275	1	Homozygous	23538339	
		NM_000551.3: c.429C>T	p.(Asp143Asp)	rs773556807	2	Homozygous	29891534 (51)	
		NM_000551.3: c.430G>A	p.(Gly144Arg)	rs869025650	2/163	Compound heterozygous	24115288 (4)	Together with variant p.(Arg200Trp) or p.(Pro81Ala)
					1/6	Heterozygous	15921386	Stop gained variant
	Exon 3	NM_000551.3: c.524A>G (523A>G)	p.(Tyr175Cys)	rs193922613	1/5	Heterozygous	15642680	
		NM_000551.3: c.548C>T	p.(Ser183Leu)	rs5030823	1	Heterozygous	21454469	
		NM_000551.3: c.562C>G	p.(Leu188Val)	rs5030824	2/7	Compound heterozygous	12844285	Together with variant p.(Arg200Trp)
		NM_000551.3: c.571C>G	p.(His191Asp)	rs28940301	1/7	Homozygous	12844285	
		NM_000551.3: c.574C>T	p.(Pro192Ser)	rs28940300	1/7	Compound heterozygous	12844285	Together with variant p.(Arg200Trp)
		NM_000551.3: c.574C>A	p.(Pro192Thr)	/	1/163	Compound heterozygous	24115288 (4)	Together with variant p.(Arg200Trp)
		NM_000551.3: c.586A>G	p.(Lys196Glu)	rs281860296	1/70	Homozygous	23859443	

Revised genetic variants of the genes associated with congenital erythrocytosis (ECYT) types 1-8, as an update of the Leiden Open Variation Database.
dbSNP ID: Single Nucleotide Polymorphism Database identifier; PMID: PubMed identifier.

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Table II - *Genetic variants associated with congenital erythrocytosis (continued from previous page)*

EGLN1	**Exon 1**	NM_022051.2: c.122A>G	p.(Tyr41Cys)	/	2 / 1,192	Heterozygous	29790589	
		NM_022051.2: c.380G>C	p.(Cys127Ser)	rs12097901	8/163	Heterozygous	24115288 (4)	
		NM_022051.2: c.461C>A	p.(Ser154*)	rs1018129986	2/1,192	Heterozygous	29790589	Stop gained variant
		NM_022051.2: c.494delC	p.(Pro165Glnfs*9)	/	1/1,192	Heterozygous	29790589	
		NM_022051.2: c.494dupC	p.(Ser166Lysfs*81)	/	1/1,192	Heterozygous	29790589	
		NM_022051.2: c.610A>G (c.610G>A)	p.(Lys204Glu)	/	1/163	Heterozygous	24115288 (4)	
		NM_022051.2: c.678dupG	p.(Arg227Alafs*20)	/	3 (2 family members)	Heterozygous	27774468 (55)	
		NM_022051.2: c.682G>T	p.(Ala228Ser)	/	1/2	Heterozygous	25263965	Comorbidity of phaeochromocytoma (PHEO) /paraganglioma (PGL)
		NM_022051.2: c.715C>T	p.(Gln239*)	/	1		27774468 (55)	
		NM_022051.2: c.815T>C	p.(Leu272Pro)	/	1/1,192	Heterozygous	29790589	
		NM_022051.2: c.835_850del (c.835del14)	p.(Leu279Thrfs*43)	/	1/38	Heterozygous	24482100	
		NM_022051.2: c.836T>C	p.(Leu279Pro)	/	1/125	Heterozygous	27651169 (10)	
		NM_022051.2: c.853G>C	p.(Gly285Arg)	rs1184568745	1/163	Heterozygous	24115288 (4)	
		NM_022051.2: c.867C>G	p.(Ser289Arg)	rs763045676	1/1,192	Heterozygous	29790589	
	Exon 2	NM_022051.2: c.896T>C	p.(Met299Thr)	/	2/1,192	Heterozygous	29790589	
		NM_022051.2: c.911C>T	p.(Pro304Leu)	rs1293106237	1		27774468 (55)	
		NM_022051.2: c. 949_950delinsAG	p.(Pro317Arg)	/	1/1,192	Heterozygous	29790589	
		NM_022051.2: c.1000T>C	p.(Trp334Arg)	/	5 (family members)	Heterozygous	23859443	Damaging effect on protein function (*In silico* predictions)
		NM_022051.2: c.1001G>A	p.(Trp334*)	/	1/32		27034858	
	Exon 3	NM_022051.2: c.1012dup (c.1010dup)	p.(Val338Glyfs*18)	/	1/163	Heterozygous	24115288 (4)	
		NM_022051.2: c.1030C>T	p.(Arg344*)	rs752961498	1/1,192	Heterozygous	29790589	Stop gained variant
		NM_022051.2: c.1096T>C	p.(Phe366Leu)	/	2 (family members)	Heterozygous	29881576 (53)	
		NM_022051.2: c.1111C>T	p.(Arg371Cys)	/	1		27774468 (55)	
		NM_022051.2: c.1132C>T	p.(Pro378Ser)	rs1437849917	1/1,192	Heterozygous	29790589	
	Exon 4	NM_022051.2: c.1153G>A	p.(Ala385Thr)	/	2/1,192	Heterozygous	29790589	
		NM_022051.2: c.1167G>T	p.(Trp389Cys)	/	1/1,192	Heterozygous	29790589	
EPAS1	**Genetic variants are listed in Kristan *et al.* (2019)[38]**							
EPO	**Genetic variants are listed in Vočanec *et al.* (2019)[59]**							
HBB	**Exon 2**	NM_000518.4:c.310T>C	p.(Phe104Leu)	/	1	Heterozygous	28332377 (88)	
	Exon 3	NM_000518.4:c.412G>A	p.(Val138Met)	rs748704616	1/38	Heterozygous	24482100	
BPGM	**Exon 3**	NM_199186.2:c.184C>T	p.(Arg62Trp)	rs1436218818	1/1,192	Heterozygous	29790589	
		NM_199186.2:c.344G>A	p.(Trp115*)	rs149329328	1/1,192	Homozygous	29790589	Stop gained variant

Revised genetic variants of the genes associated with congenital erythrocytosis (ECYT) types 1-8, as an update of the Leiden Open Variation Database.
dbSNP ID: Single Nucleotide Polymorphism Database identifier; PMID: PubMed identifier.

leading to erythrocytosis. An *EPOR* variant in exon 8 leads to the appearance of a new C-terminal tail that increases EPOR dimerisation, constitutive signalling, and hypersensitivity to EPO[47].

Oxygen-sensing defects in familial erythrocytosis types 2-5

Familial erythrocytosis types 2-5 (ECYT2-5) are associated with genes that affect the HIF-EPO signalling pathway, including *VHL* (ECYT2), *EGLN1* (ECYT3), *EPAS1* (ECYT4), and *EPO* (ECYT5)[32,72,73,81,82].

The disease mechanism underlying these variants is inactivation of the EGLN1 and VHL enzymes, EPAS1 stabilisation, or increased EPO transcription activation, all of which result in increased EPO production under normoxic conditions and, consequently, RBC overproduction. The promoter and enhancer regions surrounding the respective genes might also represent important variant sites[51,58,59].

The *VHL* variants that cause ECYT2, an autosomal recessive disorder, lead to loss of function and can be homozygous, heterozygous, or compound heterozygous. Over 15 variants associated with ECYT2 are spread all over the coding regions of the gene. Recently, a variant in intron 1 which results in splicing alterations was linked with the disease[51]. Chuvash polycythaemia with variant *VHL* c.598C>T (p.Arg200Trp) is the most common form of ECYT2[55,83,84]. Variants can also result in angiogenesis and other changes in metabolism[51]. *VHL* is a tumour suppressor gene, so its loss-of-function variants can also result in highly vascularised tumours in the central nervous system, retinal haemangioblastomas, pancreatic neuroendocrine tumours, phaeochromocytomas, and clear-cell renal carcinomas[51].

ECYT3 is associated with more than 15 heterozygous loss-of-function EGLN1 variants near to or within the prolyl hydroxylase domain. It appears that specific variants in the MYND type zinc finger can also cause erythrocytosis[54].

Most of the nine *EPAS1* variants that cause ECYT4, an autosomal dominant disorder, are heterozygous variants of exon 12, which encode the oxygen-dependent degradation domain. These variants prevent the binding of EGLN1 hydroxylase to the EPAS1 protein and subsequent hydroxylation and binding of the VHL protein[38,81].

EPO was added to the list of ECYT-causing genes most recently, which led to the definition of an autosomal dominant disorder ECYT5. It is known that two frameshift variants interrupt the transcription of the main *EPO* mRNA, which results in excess production of EPO through an alternative promoter in intron 1[58,59].

Haemoglobin-oxygen affinity defects in familial erythrocytosis types 6-8

Familial erythrocytosis types 6-8 (ECYT6-8) are linked to variants in genes that affect the affinity of haemoglobin for oxygen, including different haemoglobin subunits *HBB* (ECYT6), *HBA1*, and *HBA2* (ECYT7) and the haemoglobin-modifying enzyme *BPGM* (ECYT8).

The autosomal dominant disorders ECYT6 and ECYT7 are caused by heterozygous variants in *HBB* and *HBA* that result in high oxygen affinity of haemoglobin[85-87]. The *HBB* gene variants (over 80 variants) are more frequent than those of *HBA1* and *HBA2* (over 24 variants)[88]. The three crucial regions that are responsible for haemoglobin stability are its β-chain C terminus, the α1β2 interface, and the 2,3-BPG) binding site[85]. *HBB* variants with high oxygen affinity affect the transition of the R-state (relaxed binding structure) to the T state (tight-binding structure). Variants in the 2,3-BPG binding site and the HBB haem pocket have also been reported. Substitutions are the major cause of defects in the function of HBA. A small proportion of survivable transferable variants has also been attributed to deletions and frameshift variants[88].

The mechanism of erythrocytosis of the haemoglobin variants is based on lower concentrations of free oxygen, resulting in tissue hypoxia and increased EPO production. Haemoglobin variants can result in further pathologies, such as sickle cell anaemia, thalassaemia, and unstable haemolytic anaemias.

Rare compound heterozygous variants in *BPGM* reported to be associated with ECYT8 are the result of a BPGM deficiency due to mutations in proximity to active binding sites. Up to now, several variants have been found, although only four variants are known to cause ECYT8[44,89-91].

STEPWISE CLINICAL EXAMINATION AND GENETIC TESTING

Clinical examination

Normally a patient suspected of having erythrocytosis is referred to a haematologist by a general practitioner with a few old complete blood count results. A stepwise clinical

and laboratory assessment follows[4,5,92]. It is a good decision to measure RCM at this point if it is possible to do this investigation. It is essential to take the patient's history and conduct a thorough physical evaluation, focusing on symptoms, comorbidities, medications taken, habits, and family history. Diagnostic predictions are then followed by laboratory evaluation.

High haematocrit, haemoglobin concentration, and/or RCM for >2 months with an appropriate clinical picture and biochemistry confirm absolute erythrocytosis (first step). In the second step, acquired secondary erythrocytosis due to chronic diseases (heart, lung, kidney) and other conditions (smoking, sleep apnoea, therapy with steroids, or their misuse) should be excluded. The important third step is to determine whether the patient has PV by measuring serum EPO levels and screening for *JAK2* variants. A single point *JAK2* variant V617F indicative of PV can be identified by allele-specific polymerase chain reaction (PCR) analysis[93]. Cases without *JAK2* V617F should be screened for exon 12 variants by using the melting curve assay[63]. In the fourth step, genes related to *ECYT* are sequenced. When the EPO level is low, usually only the *EPOR* gene is sequenced; otherwise, the testing focuses on the *HBB*, *HBA*, *BPGM*, *VHL*, *EPAS1*, *EGLN1*, and *EPO* genes. If no known causative variant is found in these genes, extended NGS analysis should be performed[6,60]. A remaining group of patients with unexplained erythrocytosis is characterised as having idiopathic erythrocytosis.

Genetic testing

Different techniques are used for the detection of variants in the selected genes. Depending on the gene variant, the whole gene or specific regions of the gene can be sequenced. The most common analysis is PCR amplification of the targeted region, followed by Sanger sequencing[94,95]. Techniques such as comparative genomic hybridisation, quantitative PCR, and high resolution melting are also used[41]. However, NGS has gained ground here over the last decade, due to the possibility of analysing a large number of samples and genes in one test[10,96,97]. Nevertheless, Sanger sequencing is still used for validation of the NGS results or characterisation of additional members from the same ECYT family.

One of the most promising NGS approaches, besides whole-exome sequencing and whole-genome sequencing, is targeted gene sequencing with a *NGS* gene panel. The selection of the specific genes involved in the disease development increases the coverage and reduces the costs of targeted gene sequencing, compared to whole-genome/exome sequencing. Customised NGS panels are constantly evolving through the addition of new candidate genes to the currently known gene list (**Table I**). An important step in the process enabling extraction of rare genomic and potential erythrocytosis-causing variants is variant annotation. Since NGS technology is becoming more affordable, it is now possible to precisely screen more samples cost-effectively and detect multiple variants across different genes in less time.

Different erythrocytosis gene panels are now available for the detection of one or more types of erythrocytosis (see **Table III**). Along with the common genes involved, the NGS gene panels enable analysis of additional genes, depending on the specific needs and supplier's options. Following clinical trials, several additional new gene panels that are awaiting approval can be used to sequence the *JAK2*, *EPO*, *HBB*, *HBA1*, and *HBA2* genes (see ClinicalTrials.gov). The US Food and Drug Administration has approved a PV test that detects the *JAK2* gene using PCR[98]. In the European Union, a panel for the detection of erythrocytoses has been approved which includes the *EPOR*, *VHL*, *EGLN1*, *EPAS1*, *HBB*, *HBA1*, *HBA2*, *BPGM*, *PKLR*, *HIF1A*, *EGLN2*, and *EGLN3* genes[99].

The National Center for Biotechnology Information (NCBI) Genetic Testing Registry Site contains information about 92 genetic tests for erythrocytosis. These tests can detect one or more specific types of erythrocytosis in a group with other clinically significant diseases. The different companies diagnose erythrocytosis through the use of different sets of genes[100].

Potential new candidate genes involved in erythrocytosis

With current NGS testing, over 70% of patients suspected of having familial erythrocytosis remain undiagnosed[10,18,60]. Therefore, several other genes must influence the onset of erythrocytosis. The most probable candidate genes are from pathways that alter EPO expression, regulate the EPO-EPOR signalling pathway, and influence erythrocyte

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production, function, and apoptosis. Their function may be direct or indirect, through their roles as enzymes, transporters, inhibitors, transcription factors, and other DNA and RNA interacting proteins. A detailed search of public databases (NCBI, GenCards, UniProt, Reactome, WikiPathways, etc.) could be performed in the future with the aim of identifying new candidate genes suitable for inclusion in extended targeted NGS.

Table III - *List of diagnostic tests for erythrocytosis*

Company[§]	Test*	Genes (including)	Technique
Ambry Genetics	*VHL* gene sequence and deletion/ duplication	*VHL*	NGS, MLPA
	RenalNext	19 genes (*VHL*)	NGS
	PGLNext	12 genes (*VHL*)	NGS
Baylor Miraca Genetics Laboratories	*VHL* comprehensive - sequence and deletion/ duplication analysis	*VHL*	Sanger, MLPA
	VHL deletion/ duplication analysis (different tests)	*VHL*	MLPA, Sanger
	Hereditary paraganglioma/ pheochromocytoma panel	9 genes (*VHL*)	NGS, MLPA
Bioarray	Familial erythrocytosis	*EPOR*	NGS
BloodGenetics	NGS panel for congenital erythrocythosis or familiar polycythaemia	*BPGM*, *EGLN1*, *EPAS1*, *EPOR*, *JAK2*, *SH2B3*, *VHL*	NGS
CeGaT GmbH	Erythrocytes, anaemia panel	*AMN*, *ANK1*, *C15orf41*, *CBLIF*, *CDAN1*, *COX4I2*, *CUBN*, *EGLN1*, *EPAS1*, *EPB42*, *EPOR*, *G6PD*, *HBA1*, *HBA2*, *HBB*, *HBD*, *HFE*, *KIF23*, *KLF1*, *LPIN2*, *RPL11*, *RPL35A*, *RPL5*, *RPS10*, *RPS17*, *RPS19*, *RPS24*, *RPS26*, *RPS7*, *SEC23B*, *SH2B3*, *SPTA1*, *SPTB*	NGS/MPS
	Single gene testing *JAK2*	*JAK2*	Sanger
	Single gene testing *VHL*	*VHL*	Sanger
CEN4GEN Institute for Genomics and Molecular Diagnostics	Primary familial and congenital polycythaemia: gene sequencing	*EPOR*	NGS/MPS
Center for Human Genetics	Hereditary onco-endocrine tumours	44 genes (*EGLN1*, *EPAS1*, *VHL*)	NGS/MPS
Centogene AG - the Rare Disease Company Germany	Erythrocytosis, familial type 1	*EPOR*	qPCR, Sanger
	Erythrocytosis, familial type 1	*SH2B3*	qPCR, Sanger
	Erythrocytosis, familial type 3	*EGLN1*	qPCR, Sanger
	Erythrocytosis, familial type 4	*EPAS1*	qPCR, Sanger
CGC Genetics	Erythrocytosis, familial (sequence analysis of *EPOR* gene)	*EPOR*	Sanger
	Erythrocytosis familial, 3 (sequence analysis of *EGLN1* gene)	*EGLN1*	Sanger
	Erythrocytosis familial, 4	*EPAS1*	Sanger
	Detection of V617F somatic mutation of *JAK2* gene	*JAK2*	RT-qPCR
	Pheochromocytoma and paraganglioma (NGS panel for 16 genes)	*EGLN1*, *FH*, *GDNF*, *KIF1B*, *MAX*, *MEN1*, *NF1*, *PRKAR1A*, *RET*, *SDHA*, *SDHAF2*, *SDHB*, *SDHC*, *SDHD*, *TMEM127*, *VHL*	NGS/MPS
Cincinnati Children's Hospital Medical Center Laboratory of Genetics and Genomics	Erythrocytosis panel	*BPGM*, *EGLN1*, *EPAS1*, *EPOR*, *HBA1*, *HBA2*, *HBB*, *JAK2*, *VHL*	NGS
	Thrombocytosis panel by NGS	*CALR*, *JAK2*, *MPL*, *THPO*	NGS
	EPOR sequencing	*EPOR*	Sanger
	EPAS1 sequencing	*EPAS1*	Sanger

[§]See Appendix 1; *Tests names are protected under a trademark owned by a business company.

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Table III - *List of diagnostic tests for erythrocytosis (continued from previous page)*

Company[§]	Test*	Genes (including)	Technique
Fulgent Genetics	Erythrocytosis NGS panel	*ANK1*, *BPGM*, *CALR*, *EGLN1*, *EPAS1*, *EPB41*, *EPB42*, *EPOR*, *HBA1*, *HBA2*, *HBB*, *HIF1A*, *JAK2*, *KCNN4*, *PIEZO1*, *PKLR*, *RHAG*, *SH2B3*, *SLC4A1*, *SPTA1*, *SPTB*, *TET2*, *TET3*, *VHL*	NGS
	Clinical exome	4,673 genes for 5156 conditions (*VHL*, *EGLN1*, *EPAS1*)	NGS
	Full comprehensive cancer panel	127 genes for 329 conditions (*VHL*, *EGLN1*)	NGS/MPS
	Comprehensive thrombosis, platelet disorder and coagulation deficiency NGS panel	82 genes for 146 conditions (*EGLN1*, *EPAS1*)	NGS
	Von Hippel-Lindau syndrome (*VHL* single gene test)	*VHL*	NGS/MPS
	Expanded polycystic kidney disease NGS panel	34 genes (*VHL*)	NGS/MPS
	Comprehensive primary immunodeficiency NGS panel	472 genes (*SH2B3*, *JAK2*)	NGS
	SPRTN single gene	*SPRTN*	NGS/MPS
	Paraganglioma-pheochromocytoma comprehensive panel	11 genes (*VHL*)	NGS/MPS
	EPOR single gene	*EPOR*	NGS/MPS
	VHL single gene	*VHL*	NGS/MPS
	SH2B3 single gene	*SH2B3*	NGS/MPS
	JAK2 single gene	*JAK2*	NGS/MPS
	Myelofibrosis NGS panel	*CALR*, *JAK2*, *MPL*, *SH2B3*	NGS/MPS
	EGLN1 single gene	*EGLN1*	NGS/MPS
GeneDx	*VHL* gene sequencing	*VHL*	Sanger
GENETAQ Molecular Genetics Centre and Diagnosis of Rare Diseases	Erythrocytosis, familial: *EPOR* gene sequence analysis	*EPOR*	Sanger
	Erythrocytosis, familial: *EGLN1* gene sequence analysis	*EGLN1*	Sanger
	Erythrocytosis, familial: *EPAS1* (*HIF2A*) gene sequence analysis	*EPAS1*	NGS
Instituto de Medicina Genomica	*EPOR*. Complete sequencing	*EPOR*	Sanger
Labor Dr. Wisplinghoff	Familial erythrocytosis, 1	*EPOR*	Sanger
Laboratorio de Genetica Clinica SL	Familial erythrocytosis	*EPOR*	Sanger
	Polycythaemia, secondary (autosomal dominant)	*EGLN1*, *EPAS1*	Sanger
	Primary familial polycythaemia	*EPOR*	Sanger
Mayo Clinic Genetic Testing Laboratories	2,3-Bisphosphoglycerate mutase, full gene sequencing analysis	*BPGM*	Sanger
MedGene	Erythrocytosis, somatic	*JAK2*	Sanger
Genome Diagnostics Lab.	*VHL* gene sequencing and deletion/duplication analysis	*VHL*	NGS, MPS, MLPA
	Custom gene sequencing panel	31 genes (*VHL*)	NGS
Praxis fuer Humangenetik Wien	Erythrocytosis, somatic	*JAK2*	Sanger

[§]See Appendix 1; *Tests names are protected under a trademark owned by a business company.

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Table III - *List of diagnostic tests for erythrocytosis (continued from previous page)*

Company[§]	Test*	Genes (including)	Technique
PreventionGenetics	Primary familial and congenital polycythaemia (PFCP) via *EPOR* gene	*EPOR*	NGS, Sanger, MPS
	Von Hippel-Lindau disease via *VHL* gene sequencing with CNV detection	*VHL*	NGS, Sanger
Reference Laboratory Genetics	Familial erythrocytosis, panel massive sequencing (NGS) four genes	*EGLN1*, *EPAS1*, *EPOR*, *VHL*	NGS
	Familial erythrocytosis, sequencing *EPOR* gene	*EPOR*	Sanger
	Familial erythrocytosis type 2, sequencing *VHL* gene	*VHL*	Sanger
	Familial erythrocytosis, sequencing *EGLN1* gene	*EGLN1*	Sanger
	Familial erythrocytosis type 4, sequencing exon 12 *EPAS1* gene	*EPAS1*	Sanger
	Familial erythrocytosis, sequencing *EPAS1* gene	*EPAS1*	Sanger
	Erythrocytosis due to bisphosphoglycerate mutase deficiency, sequencing *BPGM*	*BPGM*	Sanger

[§]See Appendix 1; *Tests names are protected under a trademark owned by a business company. Tests names may change, products can be removed from the market. The National Center for Biotechnology Information (NCBI) Genetic Testing Registry Site contains information about 92 genetic tests for the detection of erythrocytosis alone plus other groups of clinically important diseases. MLPA, multiplex ligation-dependent probe amplification; CGH, comparative genomic hybridisation; MPS, massive parallel sequencing; NGS, next-generation sequencing; qPCR, quantitative polymerase chain reaction; Sanger, sequencing by Sanger; RT-qPCR, quantitative reverse transcription-polymerase chain reaction; CNV: copy number variation.

CONCLUSIONS

The purpose of this review was to describe the current, rapidly evolving knowledge in the wide and diverse field of erythrocytosis. A group of patients with idiopathic erythrocytosis is of broad interest to clinicians, geneticists, and researchers. NGS techniques represent the preferred methods for the rapid discovery of new unknown variants involved in the development of erythrocytosis.

Further studies focusing on the discovery of new genes and variants that are responsible for erythrocytosis are urgently needed. In the future, several unknown causative genes are expected to be identified as indicative of different types of erythrocytosis. Major targets are the genes involved in three pathways: EPO-EPOR signalling, the HIF-EPO pathway, and haemoglobin–BPGM-regulated oxygen affinity.

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APPENDIX 1

Company	Town	State
Ambry Genetics	Aliso Viejo	USA
Baylor Miraca Genetics Laboratories	Houston	USA
Bioarray	Elche	Spain
BloodGenetics	Llobregat	Spain
CeGaT GmbH	Tuebingen	Germany
CEN4GEN Institute for Genomics and Molecular Diagnostics	Edmonton	Canada
Center for Human Genetics	Brussels	Belgium
Centogene AG - the Rare Disease Company Germany	Rostock	Germany
CGC Genetics	Porto	Portugal
Cincinnati Children's Hospital Medical Center Laboratory of Genetics and Genomics	Cincinnati	USA
Fulgent Genetics	Temple City	USA
GeneDx	Gaithersburg	USA
GENETAQ Molecular Genetics Centre and Diagnosis of Rare Diseases	Malaga	Spain
Instituto de Medicina Genomica	Paterna	Spain
Labor Dr. Wisplinghoff	Koeln	Germany
Laboratorio de Genetica Clinica SL	Madrid	Spain
Mayo Clinic Genetic Testing Laboratories	Rochester	USA
MedGene	Bratislava	Slovakia
Genome Diagnostics Lab	Toronto	Canada
Praxis fuer Humangenetik Wien	Wien	Austria
PreventionGenetics	Marshfield	USA
Reference Laboratory Genetics	Llobregat	Spain

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