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Original Article

Coexistence of Multiple Gene Variants in Some Patients with Erythrocytoses

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Abstract. ***Background*: Erythrocytosis is a relatively common condition; however, a large proportion of these patients (70%) remain without a clear etiologic explanation.**
***Methods*: We set up a targeted NGS panel for patients with erythrocytosis, and 118 sporadic patients with idiopathic erythrocytosis were studied.**
***Results*: In 40 (34%) patients, no variant was found, while in 78 (66%), we identified at least one germinal variant; 55 patients (70.5%) had 1 altered gene, 18 (23%) had 2 alterations, and 5 (6.4%) had 3. An altered *HFE* gene was observed in 51 cases (57.1%), *EGLN1* in 18 (22.6%) and *EPAS1*, *EPOR*, *JAK2*, and *TFR2* variants in 7.7%, 10.3%, 11.5%, and 14.1% patients, respectively. In 23 patients (19.45%), more than 1 putative variant was found in multiple genes.**
***Conclusions*: Genetic variants in patients with erythrocytosis were detected in about 2/3 of our cohort. An NGS panel including more candidate genes should reduce the number of cases diagnosed as "idiopathic" erythrocytosis in which a cause cannot yet be identified. It is known that *HFE* variants are common in idiopathic erythrocytosis. *TFR2* alterations support the existence of a relationship between genes involved in iron metabolism and impaired erythropoiesis. Some novel multiple variants were identified. Erythrocytosis appears to be often multigenic.**

Keywords: Next generation sequencing; Germline variant; Erythrocytosis; Iron metabolism.

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Introduction. Erythrocytosis, characterised by increased haemoglobin (Hb) and/or haematocrit (HT) levels, is a relatively common condition in the general population;[1] some of these patients are affected by polycythaemia vera (PV);[2] acquired secondary erythrocytosis are more frequent and are due to increased levels of erythropoietin (EPO) appropriately or inappropriately produced. Finally, congenital erythrocytosis may be the consequence of mutations of the globin genes (*HBB* or *HBA*), or bi-phosphoglycerate mutase (*BPGM*),[3] or of gene mutations involved in the oxygen sensing pathway (*VHL*, *PHD2, HIF2A*) or of the erythropoietin receptor gene (*EPOR*).[4]

Unfortunately, a large proportion (70%) of patients with erythrocytosis remains without a clear aetiologic explanation[5] and, consequently, the condition has been classified as idiopathic erythrocytosis (IE).[6]

We observed that a significant percentage of IE patients have high or borderline high ferritin levels, suggesting that at least in some of the patients, erythrocytosis coexists with an impaired iron balance, and *HFE* gene mutations are higher in IE than in the general population.[7]

The availability of next-generation sequencing

(NGS) facilities in our laboratory prompted us to build a specific gene panel to search for novel variants in patients with erythrocytosis of uncertain aetiology.[8–10]

Material and Methods. From 2013 to 2020, in our centre, we encountered 118 (M/F =101/17; mean age 53.7±17.2 years) patients who were diagnosed with idiopathic erythrocytosis. Patients' inclusion criteria were: (i) haemoglobin >180 g/L and haematocrit >52% in males and haemoglobin >160 g/L and haematocrit >46% in females, (ii) long-standing unexplained erythrocytosis, (iii) absence of relatives with increased Hb or HT, (iv) no evidence of smoke, arterial-venous shunt, pulmonary and/or renal diseases or neoplasms, (v) absence of somatic mutations in *JAK2* (p.Val617Phe or variants in *JAK2* exon 12). Patients carrying haemoglobin variants with high oxygen affinity, as evaluated by venous $p50^1$ and those with a left-shifted oxygen dissociation curve[11] were excluded. No children younger than 16 years of age are included in the present cohort, as our surgery is dedicated only to adults. We evaluated nine normal subjects (normal Hb/HT values and no clinical signs of erythrocytosis) as controls.

The patients' data were collected in an ad hoc performed anonymous database approved by the Ethics Committee (EC) of the General Hospital-University of Padova, Italy. The patients gave consent to data collection for scientific purposes and publication. The study was conducted in accordance with the Declaration of Helsinki.

Thrombotic and haemorrhagic events were recorded. We collected red blood cell (RBC) counts, Hb and HT levels in all patients, ferritin levels in 67, transferrin saturation (T-Sat) in 38, and serum erythropoietin (EPO) in 66.

According to the manufacturer's instructions, DNA was extracted from granulocytes obtained from patients' blood samples using EuroGold Blood DNA Mini Kit Plus (EuroClone) and stored at -20°C. DNA concentration was determined by Qubit 4 Fluorometer with Qubit™ 1X dsDNA HS Assay Kits (Thermo Fisher Scientific).

A targeted NGS panel for patients with erythrocytosis was set up. The panel included the coding sequence of 14 genes (*BPGM, EGLN1, EPAS1, HAMP, FTL, HFE, HFE2, JAK2, SLC11A2, SLC40A1, TFR2, VHL, FTH1, EPOR*) involved in erythrocytosis because we choose to obtain a targeted sequence of genes interested in a specific group of patients. The panel is an "On Demand AmpliSeq Panel" designed with the Illumina Design Studio platform and validated in silico by Illumina. Libraries were prepared by using "AmpliSeq for Illumina On-Demand, Custom, and Community Panels" (Illumina) as the manufacturer's instructions. The sequencing was performed in a MiSeq Illumina instrument (151 cycles paired-end; mean target coverage 500X for each sample). FASTAQ data were analysed by Illumina BaseSpace softwares and by CLC Genomics Workbench Qiagen tools. *Homo sapiens* genome assembly GRCh37 (hg19) was used as a reference, and Integrative Genomics Viewer (IGV) was used to visualise reads alignment. Germline and somatic variants were filtered using two independent and different workflows primarily based on different frequency thresholds for variant identification. In both workflows, the selection of variants was carried out through multiple filters that mainly consider the total coverage of the fragment of interest, the quality and the correct and minimum number of base calls. Only non-synonymous variants that passed all filters were considered for further validation and analysis. Germline variants identified by NGS were confirmed by direct Sanger sequencing.

Amplification conditions and primers are available upon request. The clinical significance of the identified variants was inferred using ClinVar, the OMIM database, MobiDetails, and gnomAD, according to ACMG guidelines[12] with the help of VARSOME.[13] The statistical analysis was performed by comparing the data obtained in patients and controls with a Mann-Whitney U test (two groups).

Results. At the time of the study, the patients' median Hb was 172 g/L (range 142-206) and HT 51% (range 45.7-57.2). While in 40 (34%) patients, we did not detect any gene variant, in 78 (66%), we identified at least one germinal variant in one of the studied genes (**Table 1**). Out of the 78 patients with germinal variants, 55 (70.5%) had 1 altered gene, 18 (23.1%) had 2 alterations, and 5 (6.4%) had 3. We did not find significant differences in the laboratory data and clinical characteristics between patients with or without alterations, and the number of variants had no impact on patients' parameters. The main clinical and laboratory data of patients with an undetected variant and patients with a single variant are summarised in **Table 2**.

The most common altered gene in our cohort was *HFE*, present in 51 cases (65.3%) and, in particular, the presence of *HFE*-p.His63Asp was found in 41 (52.5% of all patients carrying variants). The altered *EGLN1* gene was found in 18 (23.1%) patients, where p.Cys127Ser was the prevalent one (11 cases =61.1%). *EPAS1, EPOR*, *JAK2*, and *TFR2* variants were observed in 6 (7.7%), 8 (10.3%), 9 (11.5%), and 11 (14.1%) patients, respectively (**Table 3**).

In the remaining 23 patients (22 males and 1 female, years range 20-82), representing 19.45% of the total cohort, more than 1 putative variant was found in multiple genes (**Table 4**).

In the control group, 7 subjects (78%) carry gene variants (4 *EGLN1*, 2 *EPAS1*-p.Thr766Pro, 2 *JAK2*-p.Leu393Val, 1 *EPOR*-p.Gly46Glu). Remarkably, no normal case displayed any *HFE* or *TFR2* variant.

Table 1. Description of all molecular variants found in our patients with clinical diagnosis of idiopathic erythrocytosis.

Gene / Chr	Coding Region Change	Protein Change	Exon	rs code	Domain (D) / Topology (T)	ClinVar Prediction	gnomAD Prediction	ACMG Classification	MobiDetails Prediction
EGLN1 (PHD2) Chr1	c.380C>G	p.Cys127Ser / C127S	1	rs12097901	/	Benign	Benign	Benign	Benign
	c.471C>G	p.Gln157His / Q157H	1	rs61750991	/	Benign	Benign	Benign (Moderate)	Likely Benign
	c.806T>C	p.Ile269Thr / I269T	3	rs541542280	D: Fe2OG dioxygenase	Uncertain Significance	Uncertain Significance	Uncertain Significance	Pathogenic
	c.1108C>G	p.Arg370Gly / R370G	1	/	/	/	/	Uncertain Significance	Uncertain Significance
EPAS1 (HIF-2α) Chr2	c.1121T>A	p.Phe374Tyr / F374Y	9	rs150797491	/	Conflicting Interpretations	Conflicting Interpretations	Likely Benign	Uncertain Significance
	c.2296A>C	p.Thr766Pro / T766P	15	rs59901247	/	Benign	Benign	Benign (Moderate)	Benign
	c.1648C>T	p.Arg550Trp / R550W	12	rs771840848	/	Uncertain Significance	Uncertain Significance	Uncertain Significance	/
VHL Chr3	c.74C>T	p.Pro25Leu / P25L	1	rs35460768	/	Conflicting Interpretations	Conflicting Interpretations	Benign (Moderate)	Uncertain Significance
EPOR Chr19	c.296C>T	p.Ala99Val / A99V	3	rs146235694	T: Extracellular	Benign / Likely Benign	Benign / Likely Benign	Likely Benign (Moderate)	/
	c.1194T>G	p.Asp398Glu / D398E	8	/	T: Cytoplasmic	/	/	Uncertain Significance	/
	c.543G>C	p.Glu181Asp / E181D	4	/	D: Fibronectin type-III / T: Extracellular	/	/	Uncertain Significance	/
	c.1460A>G	p.Asn487Ser / N487S	8	rs62638745	T: Cytoplasmic	Benign / Likely Benign	Benign / Likely Benign	Benign (Moderate)	Likely Benign
	c.137G>A	p.Gly46Glu / G46E	2	rs45516306	T: Extracellular	Benign	Benign	Benign (Moderate)	Likely Benign
JAK2 Chr9	c.1177C>G	p.Leu393Val / L393V	9	rs2230723	/	Conflicting Interpretations	Conflicting Interpretations	Benign (Moderate)	Likely Benign
	c.143G>A	p.Gly48Glu / G48E	3	rs143227399	D: FERM	Conflicting Interpretations	Conflicting Interpretations	Likely Benign (Moderate)	Likely Pathogenic
	c.337C>G	p.Leu113Val / L113V	4	rs143103233	D: FERM	Likely Benign	Likely Benign	Likely Benign (Moderate)	Likely Pathogenic
	c.1711G>A	p.Gly571Ser / G571S	13	rs139504737	D: Protein kinase 1	Conflicting Interpretations	Conflicting Interpretations	Uncertain Significance	Likely Pathogenic
	c.3323A>G	p.Asn1108Ser / N1108S	25	rs142269166	D: Protein kinase 2	Conflicting Interpretations	Conflicting Interpretations	Uncertain Significance	Likely Pathogenic
	c.233C>T	p.Thr78Ile / T78I	4	/	D: FERM	/	/	Uncertain Significance	/
	c.2767C>T	p.Arg923Cys / R923C	21	rs774355597	D: Protein kinase 2	Uncertain Significance	Uncertain Significance	Uncertain Significance	/
HFE Chr6	c.187C>G	p.His63Asp / H63D	2	rs1799945	T: Extracellular	Conflicting Interpretations	Conflicting Interpretations	Pathogenic	Uncertain Significance
	c.845G>A	p.Cys282Tyr / C282Y	4	rs1800562	D: Ig-like C1-type / T: Extracellular	Conflicting Interpretations	Conflicting Interpretations	Likely Pathogenic (Moderate)	Pathogenic
	c.193A>T	p.Ser65Cys / S65C	2	rs1800730	T: Extracellular	Conflicting Interpretations	Conflicting Interpretations	Benign	Uncertain Significance
	c.829G>A	p.Glu277Lys / E277K	4	rs140080192	D: Ig-like C1-type / T: Extracellular	Conflicting Interpretations	Conflicting Interpretations	Benign (Moderate)	Likely Pathogenic
TFR2 Chr7	c.2255G>A	p.Arg752His / R752H	18	rs41295942	T: Extracellular	Benign / Likely Benign	Benign / Likely Benign	Benign	Likely Benign
	c.545C>A	p.Ala182Glu/ A182E	4	/	T: Extracellular	/	/	Uncertain Significance	Uncertain Significance
	c.1942G>T	p.Asp648Tyr / D648Y	16	/	T: Extracellular	/	/	Uncertain Significance	/

	c.1804delG	p.Asp602fs / D602fs	16	/	T: Extracellular	/	/	Uncertain Significance	/
	c.381C>A	p.Asp127Glu / D127E	3	rs145795884	T: Extracellular	Uncertain Significance	Uncertain Significance	Uncertain Significance	Uncertain Significance

Table 2. Main clinical and median laboratory data of patients with a single or no variant. For each parameter, the range is given in parentheses.

Genes	N°/Sex	Median age [years]	RBC [x10^{12}/L]	Hb [g/L]	HT [%]	Ferritin [µg/L]	T-Sat [%]	Serum EPO [IU/L]	Clinical events
No variants	33/M 7/F	58 (9-79)	5.6 (5.3-6.2)	M: 171 (157-192) F: 159 (153-165)	M: 51.3 (46-53) F: 48 (46-52)	179 (11-606)	25.2 (11-36.6)	8.05 (3.1-15)	MI in 1 patient
EGLN1	6/M 1/F	55.5 (29-74)	5.71 (5.4-6.1)	M:171 (162-187) F: 158	M:51 (46-55.8) F:49	92 (18-340)	30 (29-31.3)	6.5 (5.4-15)	None
VHL	1/F	72	5.2	163	67	194	28.3	10.4	None
EPOR	4/M	46 (29-68)	5.58 (5.4-5.7)	170 (168-178)	51.25 (50.5-52)	390.5 (63-718)	49	5.6 (4.2-7)	None
JAK2	3/M	53.5 (22-60)	5.1	174	52.5	174	-	7.7	None
HFE	27/M 7/F	59.5 (32-91)	5.6 (5.3-6.2)	M: 171 (161-195) F: 162 (157-165)	M: 50.65 (46.7-56.9) F: 50 (48-53)	199 (25-803)	29.17 (16-426.53)	11 (3.9-38)	Stroke in 1 patient
TFR2	6/M	49 (20-59)	5.56 (5.3-6.0)	174 (166-177)	52.1 (49.4-54.8)	150 (74-457)	20 (11.78-30.95)	9.1 (5.6-11.3)	None

M = Male, F = Female, N°= Number, RBC = Red Blood Cells, Hb = Haemoglobin (normal range males 135g/L-180g/L and normal range females 120g/L-150g/L), HT = Haematocrit (normal range males 40%-47% and normal range females 37%-45%), T-Sat = Transferrin Saturation, EPO = Erythropoietin, MI = Myocardial Infarction.

Table 3. Distribution and allele frequency of different molecular variants in our cohort.

Gene	Protein change	N° of each variant	Variants		Relative allele frequency in our cohort	European (non-Finnish) allele frequency gnomAD	p	Citations
			Isolated	Associated				
EGLN1	C127S	11	3	8	0.0932	0.0688	ns	Yes
	Q157H	6	3	3	0.0508	0.0275	ns	Yes
	R370G	1	0	1	-	Unknown	-	No
	I269T	1	1	0	-	Unknown	-	No
	Total	19	7 (36.8%)	12 (63.2%)				
EPAS1	F374Y	4	0	4	0.0339	0.00595	< 0.01	Yes
	T766P	1	0	1	-	0.0165	-	Yes
	R550W	1	0	1	-	0.00000879	-	No
	Total	6	0 (0%)	6 (100%)				
VHL	P25L	3	1	2	0.0254	0.00509	< 0.05	Yes
	Total	3	1 (33.3%)	2 (66.7%)				
EPOR	A99V	1	0	1	-	0.00144	-	Yes
	D398E	1	1	0	-	Unknown	-	No
	E181D	1	0	1	-	Unknown	-	No
	N487S	4	3	1	0.0339	0.00689	< 0.01	Yes
	G46E	1	0	1	-	0.00661	-	Yes
	Total	8	4 (50%)	4 (50%)				
JAK2	L393V	2	0	2	0.0169	0.00504	ns	Yes

	G48E	1	0	1	-	0.00031	-	No
	L113V	1	0	1	-	0.00121	-	No
	G571S	2	2	0	0.0169	0.000637	< 0.01	Yes
	N1108S	1	0	1	-	0.00296	-	No
	T78I	1	0	1	-	Unknown	-	No
	R923C	1	1	0	-	0.0000579	-	Yes
	Total	9	3 (33.3%)	6 (66.7%)				
HFE	H63D	41 (3 homo)	27	14	0.35	0.144	< 0.01	Yes
	C282Y	10	6	4	0.085	0.0577	ns	Yes
	S65C	3	1	2	0.025	0.0153	ns	Yes
	E277K	1	1	0	-	0.000527	-	No
	Total	55	35 (63.6%)	20 (36.4%)				
TFR2	R752H	7	4	3	0.0593	0.0321	ns	Yes
	A182E	1	0	1	-	Unknown	-	No
	D648Y	1	0	1	-	Unknown	-	No
	D602fs	1	1	0	-	Unknown	-	No
	D127E	1	1	0	-	0.000636	-	Yes
	Total	11	6 (54.5%)	5 (45.5%)				

Table 4. Main clinical and biochemical data of the 23 patients with multiple gene variants.

Sex/Age	Variants	Hb [g/L]	HT [%]	Ferritin [µg/L]	Serum EPO [IU/L]	Clinical findings
M/24	*EGLN1*-p.Cys127Ser + *JAK2*-p.Leu393Val	173	49.8	1421	-	ALL
M/22	*EGLN1*-p.Cys127Ser + *HFE*-p.His63Asp	179	53	-	-	/
M/62	*EGLN1*-p.Cys127Ser + *HFE*-p.His63Asp	178	50.9	28	6	/
M/58	*EGLN1*-p.Cys127Ser / p.Arg370Gly + *HFE*-p.His63Asp	196	53.8	-	5	/
M/41	*EGLN1*-p.Cys127Ser + *EPAS1*-p.Thr766Pro	148	45.8	-	22.1	/
M/50	*EGLN1*-p.Cys127Ser + *TFR2*-p.Arg752His	173	50.7	-	-	/
M/64	*EGLN1*-p.Gln157His + T*FR2*-p.Asp648Tyr	170	51	185	20	/
M/22	*EGLN1*-p.Gln157His + *HFE*-p.His63Asp	165	53	96	18	/
M/56	*EPAS1*-p.Arg550Trp + *HFE*-p.Cys282Tyr	171	50.9	-	4.7	/
M/43	*EPAS1*-p.PheF374Tyr + *JAK2*-p.Gly48Glu	178	53	551	5.5	/
M/47	*HFE*-p.His63Asp + *EPOR*-p.Glu181Asp	164	49	415	6.6	/
M/35	*HFE*-p.His63Asp + *EPOR*-p.Gly46Glu	180	53.4	-	-	/
M/55	*JAK2*-p.Leu393Val + *HFE*-p.His63Asp	166	54	-	7.5	/
M/82	*JAK2*-p.Asn1108Ser+ *HFE*-p.Cys282Tyr	184	54.4	-	4.4	Stroke, Hemocromatosis
F/73	J*AK2*-p.Leu113Val + *TFR2*-p.Ala182Glu	172	48.85	345	4.6	/
M/76	*VHL-p.*Pro25Leu + *HFE*-p.His63Asp / p.Ser65Cys	162	49.8	91	9.9	/
M/31	*TFR2*-p.Arg752His + *HFE*-p.His63Asp	173	49.5	191	6.6	/
M/56	*EGLN1*-p.Gln157His + *EPOR*-p.Ala99Val + *HFE*-p.His63Asp	172	49	93	22	/
M/60	*EGLN1*-p.Cys127Ser + *VHL-p.*Pro25Leu + *HFE*-p.Ser65Cys	188	53.1	93	6.05	Myocardial infarction

M/64	*EGLN1*-p.Cys127Ser + *EPAS1*-p.PheF374Tyr + *HFE*-p.His63Asp / p.Cys282Tyr	190	56.1	510	7.4	Hemochromatosis
M/60	*EPAS1*-p.PheF374Tyr + *EPOR*-p.Asn487Ser + *HFE*-p.His63Asp	172	49	260	13.1	/
M/69	*TFR2*-p.Arg752His + *JAK2*-p.Thr78Ile + *HFE*-p.His63Asp / p.Cys282Tyr	183	59.1	559	13	/
M/20	*EPAS1*-p.PheF374Tyr + *HFE*-p.His63Asp	185	54.5	183	5.8	/

M = Male, F = Female, Hb = Haemoglobin, HT = Haematocrit, ALL = Acute Lymphoblastic Leukemia.

Discussion. In about 70% of patients with erythrocytosis, a specific aetiology remains elusive despite extensive testing.[14] Aberrant function of proteins involved in the oxygen-sensing pathway (*PHD2, HIF2A, VHL, EPO*), in *EPOR* gene, and rarely in *PIEZO1* and *SLC* genes have been found in patients with sporadic erythrocytosis.[5] Thanks to the availability of the NGS technique, we studied 118 sporadic patients with a clinical diagnosis of IE. In our cohort, however, we found a high prevalence (>80%) of patients with altered genes known or supposed to be involved in familial erythrocytosis. Camps et al.[15] found erythrocytosis-causing variants in about half of their 125 patients and some novel variants in erythrocytosis-associated genes (*LNK*) using a targeted sequencing panel that did not include genes involved in iron metabolism. The higher percentage of patients with genetic alterations in our cohort is attributable to the common finding of iron metabolism gene variants; without their contribution, the frequency of variants is like Camps' data.[15] The relevant number of patients with IE carrying *HFE* variants, whose mechanism remains to be elucidated, suggests that a relationship between molecules involved in iron regulation and erythrocytosis appears reasonably conceivable.

Oxygen sensing pathway genes. *EGLN1* variants were found in 18 patients; more than half of them carried the p.Cys127Ser variant. Such a variant is common in Tibetans, often associated with variant D4E on the same allele, as it protects them against chronic mountain sickness, characterised by erythrocytosis, hypoventilation, and pulmonary hypertension.[16] The global frequency of this variant in our cohort is 0.0932, not different than that found in the general population (gnomAD) at normoxic conditions and in low-landers; the *EGLN1*-p.Cys127Ser variant is present in 15–30% of non-Tibetan controls, with a still unclear function[17] not associated with variant D4E as in our patients (https://www.ncbi.nlm.nih.gov/snp/rs12097901#frequency_tab). Six out of the 19 patients with *EGLN1* variants (31,5%) carried *EGLN1*-p.Gln157His. This variant is mostly considered benign but described in 2 members of a family with MPN in association with *JAK2*-p.Val617Phe suggests that it should represent a disease-initiating event.[18] Neither germinal nor somatic-associated *JAK2* variants were observed in our patients. In contrast, in 3 patients the *EGLN1*-p.Gln157His variant was associated with *TFR2, HFE* or *EPOR* alterations. In 2 patients we observed *EGLN1*-p.Arg370Gly and p.Ile269Thr variants, the first considered of unknown significance and the second as pathogenic using the MobiDetails annotation platform.[19]

A large number of *EPAS1* variants have been observed and considered often benign or of uncertain significance. Some mosaic or somatic *EPAS1* variants are associated with pheochromocytoma and/or paraganglioma (PPGL),[20] while gain-of-function alterations in exon 12 of the gene are known to cause familial erythrocytosis type 4 (ECYT4).[21] One of our patients presented the p.Arg550Trp variant, which has never been previously described; interestingly, a recent article[22] identified *EPAS1*-p.Arg550Gln variant in a patient with erythrocytosis and, considering that the two variants are in the same location, they interest a critical part of the protein and are in proximity to the pathogenetic variant identified by Percy et al.,[21] we speculate that they might be clinically significant. Four patients carried *HIF2A*-p.Phe374Tyr variant disrupting interaction with the Von Hippel–Lindau tumor suppressor (pVHL) in normoxian condition and 1 patient carried p.Thr766Pro already described in familial erythrocytosis but considered benign. Interestingly, *EPAS1*-p.Phe374Tyr and partially also *EPAS1*-p.Thr766Pro were stable in normoxia and retained their interaction with the pVHL.[20] *EPAS1*-p.Phe374Tyr variant seems particularly frequent in our erythrocytotic cohort: the number of cases is small and the comparison with general population loss it significance. Larger studies are needed to confirm this data.

It has been shown that the *PHD2* mutation in Tibetan highlanders, in combination with *EPAS1* polymorphism, lowers haemoglobin level concentration.[23] The authors suggested that, in Tibetan highlanders, as well as protected from polycythaemia, this genetic condition may confer physiological advantages, and the protection from erythrocytosis could be the reflection of ameliorated HIF-regulated activities.

Three sporadic patients had the *VHL*-p.Pro25Leu variant,[24] which is neither associated with Von Hipple Lindau syndrome nor with erythrocytosis. In 2 of our 3 cases with *VHL*-p.Pro25Leu the association with *HFE*-p.Ser65Cys was also highlighted, and in one, the association with *EGLN1*-p.Cys127Ser was also found.

EPOR gene. Four of our patients carried *EPOR*-p.Asn487Ser, which was already identified in familial erythrocytosis,[4,25] and is considered to play an important role in EPO signalling. The remaining four patients with *EPOR* variants had each p.Gly46Glu and p.Ala99Val (both considered benign) and p.Glu181Asp and p.Asp398Glu (previously undescribed). Half of the patients with alterations in *EPOR* presented additional variants in the genes of the panel. Of note, 4 patients with identified variants in *EPOR* also carried the *HFE*-p.His63Asp alteration.

JAK2 gene. While *JAK2* somatic mutations are well-known driver mutations in Polycythaemia Vera (PV) and MPN,[26–29] the significance of germinal modifications of *JAK2* remains unclear, as is the case for p.Gly48Glu (who also carried *EPAS1*-p.Phe374Tyr) and p.Asn1108Ser aminoacidic changes found in two of our patients. The p.Leu393Val variant, found in 2 patients, has been previously described in 1 patient who had documented normal blood counts for decades prior to his diagnosis of PV.[30] The authors hypothesised that the p.Leu393Val variant may precede the acquisition of *JAK2*-p.Val617Phe. However, the functional significance of this germline *JAK2* variant and its putative predisposing role to PV remain unclear, though its potential to predispose to malignancy is suspected, as the germline *JAK2* variant is frequently found in diffuse large B-cell tumours. Relying on these data, these patients are being closely followed up so that the appearance of PV can be promptly recognised. In addition to the somatic *JAK2*-p.Val617Phe mutation, other germline mutations can induce MPN phenotype or isolated thrombocytosis and erythrocytosis;[30] none of such germline variants have been found in our cohort and, at present, none of our patients displayed the *JAK2*-p.Val617Phe genotype. Some novel yet undescribed germinal variants of *JAK2* were found in two cases associated with *TFR2*-p.Ala182Glu and with compound *HFE* variant plus heterozygous *TFR2*-p.Arg752His alteration.

Iron metabolism genes. *HFE*-p.His63Asp, present in two-thirds of our patients,[31,32] occurs with a significantly higher frequency than in the general population.[7] *HFE*-p.His63Asp variant is not capable of influencing the normal activity of hepcidin, the negative regulator of iron homeostasis,[33,34] but in patients with increased haematocrit levels, the variant could have a pathological effect. Fourteen patients with the *HFE*-p.His63Asp variant present also other gene variants, such as *EGLN1, EPAS1, EPOR, JAK2, VHL* and *TFR2*.

HFE-p.Cys282Tyr, p.Ser65Cys and p.Glu277Lys, both common in haemochromatosis,[33] were found in fewer patients. Three cases with compound *HFE* (2 p.Cys282Tyr/ p.His63Asp and 1 p.Ser65Cys/p.His63Asp) were variously associated with *EPAS1 EGLN1, VHL, JAK2, TFR*2. Of note, none of these cases had high serum ferritin, but all had a high haematocrit.[7] It is not clear whether the *HFE* variants represent a trigger sufficient to induce erythrocytosis, as suggested by the cases with single gene mutation and iron overload, as evidenced by increased ferritin. However, *HFE* variants are frequently observed in idiopathic erythrocytosis, and we speculate that they may somehow facilitate the increase in red blood cell mass.[7,31,35] Likewise, it is not clear whether the concomitance of iron metabolism gene variants with other gene mutations may represent a fertile ground for a putative effect on erythrocytosis.[36] Considering that iron is a critical component in the process of erythrocyte production, it may have a connection with erythrocytosis.[37] Indeed, relations between *HAMP*, *EPAS1* and *EGLN1* genes to control iron metabolism have already been observed in duodenal enterocytes.[38] Studies in other tissues will define the connection of these genes with increased erythropoiesis.

Several patients carried *TFR2* alterations, the most frequent being p.Arg752His, which is considered benign and rarer in general European populations, though at present, little is known. Our finding needs to be confirmed in other cohorts of erythrocytotic patients. We found other 3 unknown variants. This gene resulted in both single and associated variants on other genes (*EGLN1, JAK2, HFE*). *TFR2* has been identified as a component of the *EPOR* complex and is required for efficient erythropoiesis.[39] In mice, the loss of *TFR2* in the erythroid compartment accounts for increased haemoglobin, indicating a deregulated erythropoiesis: it seems that TFR2 modulates the sensitivity of erythroid cells to erythropoietin (EPO) and endogenous EPO.[40,41] To the best of our knowledge, this is the first time that a mutated *TFR2* has been found in patients with erythrocytosis, suggesting that, in some cases, *TFR2* gene variants may impair erythropoiesis. Remarkably, *TFR2* variants, not found in any of the normal cases, as *HFE* alterations, bring additional support to the potential relationship between genes involved in iron metabolism and erythrocytosis. Further studies are required to confirm this hypothesis.

Unfortunately, the available number of subjects without erythrocytosis evaluated with our NGS panel is small and inadequate to be statistically compared to our large cohort of erythrocytotic patients.

In the present study, the number of patients with variants is relatively high. However, among the total number of variants detected, 2 have a potentially pathogenic effect, and 5 are considered likely pathogenetic at least in one prediction tool.

Moreover, the gene alterations found do not have a clear link to erythrocytosis. These variants need to be further investigated to evaluate their functional effect.

The gene panel we used comprehends the coding region of each gene. However, targeted genic sequencing favoured a smaller set of data that was easier to evaluate, and we are now planning to perform whole genome sequencing in our patients.

Conclusions. The use of targeted NGS panels demonstrated that several diseases are characterised by multigenic mutations: cancers,[42–44] dyslipidemia,[45] hystiocytosis,[46] etc. Our study shows that the occurrence of unexpected multiple variants, some of which are unreported yet, is common in patients with sporadic erythrocytosis. The exact role played by these variants in erythrocytosis remains to be further elucidated.

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