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Ph.D. Thesis

CONGENITAL ERYTHROCYTOSIS ARE RARE DISORDERS WITH MANY GENES INVOLVED. FUNCTIONAL EVALUATION OF NOVEL *PHD2* AND *EPOR* MUTATIONS.

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ABSTRACT

Congenital Erythrocytosis (CE) are rare and heterogeneous clinical entities. They are caused by genetic deregulation of the erythroid production resulting in increased production of red blood cells (RBCs). Primary Congenital Familial Polycythemia (PCFP) is due to erythropoietin receptor (EPOR) mutations and is associated with reduced levels of serum erythropoietin (EPOs). Secondary CE are characterized by high EPOs levels, may be due to mutations of the oxygen-sensing pathway (OSP) genes: von Hippel-Lindau (*VHL*), hypoxia-inducible factor 2 alpha (*HIF2A/EPAS1*) and prolyl hydroxylase 2 (*EGLN1/PHD2*).

Within 106 patients followed in our centre with sporadic not myeloproliferative erythrocytosis we found 9 mutations (8,5%) in the involved genes. Here we report the functional studies of 1 novel *PHD2* (c.1045G>A) and of 2 *EPOR* gene missense mutations (c.1013G>A and c.1022C>T).

To evaluate the HIF transcriptional activity of *PHD2* mutation, an *in cellulo* reporter assay has been performed, while the hydroxylation capacity of PHD2 variants has been tested with an *in vitro* Hydroxylation test. PHD2 variant does not show a clear loss-of-function of the PHD2 proteins. More sensitive tests could be developed and other PHD2 partners may be tested in this patient.

The activation of EPOR mutated signaling was evaluated with a kinetic assay using transfected K562 cell lines. The EPOR signaling cascade results more active in mutated cells than in the WT cells when stimulated with EPO as shown by higher phosphorylation of STAT5 and ERK. Both mutations impair the C-terminal negative regulatory domain and determine gain-of-function in the EPOR signalling cascade.

These are the first missense mutations of EPOR with a functional demonstrated activity that affect the EPOR signaling cascade.

Other candidate genes need to be investigated to completely understand the ethiology of high hematocrit (HCT) level both in congenital and sporadic erythrocytosis

RIASSUNTO

Le Eritrocitosi Congenite (CE) sono entità cliniche rare ed eterogenee. Esse sono causate dalla deregolazione genetica della produzione eritroide con conseguente incremento della produzione delle cellule rosse del sangue (RBCs). La Policitemia Congenita Primaria Familiare (PCFP) è dovuta a mutazioni del recettore dell'eritropoietina (EPOR) ed è associata a bassi livelli di eritropoietina sierica (EPOs). Le CE secondarie, invece, sono caratterizzate da elevati valori di EPOs dovuti ad alterazioni dei geni dell' *Oxygen Sensing Pathway* (OSP): von Hippel-Lindau (*VHL*), *hypoxia-inducible factor 2 alpha* (*HIF2A/EPAS1*) e *prolyl hydroxylase 2* (*EGLN1/PHD2*).

Nel nostro centro sono stati seguiti 106 pazienti con eritrocitosi sporadica non mieloproliferativa nei quali abbiamo trovato 9 mutazioni (8,5%) nei geni candidati.

Al fine di valutare l'attività trascrizionale di HIF in presenza di PHD2 mutato è stato messo a punto un *reporter assay in cellulo*, mentre l'attività di idrossilazione di PHD2 è stata testata con un *hydroxylation test in vitro*. Non è stata riscontrata una chiara *loss-of-function* per la variante di PHD2 nonostante il link mutazione-malattia. Pertanto si necessita lo sviluppo di test più sensibili oltre allo studio di altri geni partner.

La cascata del segnale di EPOR mutato è stata studiata effettuando una cinetica di stimolazione su cellule K562 trasfettate. In seguito a stimolazione con EPO il *signaling* di EPOR è risultato più attivo in condizioni mutate rispetto al *wild type* rilevando alti livelli di fosforilazione di STAT5 e ERK. Entrambe le mutazioni compromettono il dominio regolatore negativo C-terminale di EPOR causando una *gain-of-function* del recettore.

Queste sono le prime mutazioni missenso in cui in cui è stata rilevata un'iperattivazione dei componenti della cascata del segnale di EPOR.

Altri geni candidati devono essere investigati per comprendere completamente l'eziologia degli alti valori di ematocrito (HTC) sia nei casi di eritrocisi sporadiche sia in quelle congenite.

INTRODUCTION

Erythrocytes (RBCs), are the main cellular component of blood: they constitute approximately one-half of blood volume. Their primary function is to transport oxygen to body tissues. Oxygen, in turn, is the terminal electron acceptor of the mitochondrial electron transport chain, which allows for the generation of high levels of ATP from glucose. The primary protein component of RBCs is the iron-containing hemoglobin (HB) molecule, which is the chief carrier of oxygen to the tissues. HB has a quaternary structure assembled by two α- and two β-peptide chains that are arranged into a tetramer form identical halves, each of which is composed of one α and one β chain. Using the heme iron moiety, oxygen is loaded in the respiratory organs and off-loaded in the other tissues of the body. During this process, the HB molecule changes its conformational state moving from a tense state to a relaxed state. The affinity of HB toward oxygen is reduced to allow oxygen to be released at sites of low oxygen tension. The affinity of HB for oxygen can be influenced by pH, pCO_2, temperature and by 2,3-bisphosphoglycerate (2,3-BPG), which binds directly to the HB molecule better than O_2. Indeed, it diminishes with the decrease of pH or increases in either pCO_2 or 2,3-BPG, which is synthesized by the enzyme 2,3-BPG mutase (BPGM).

Low concentrations of RBCs cause inefficient delivery of oxygen to the tissues, leading to the inability to meet metabolic demands. At the same time, high concentrations of RBCs can cause hyperviscosity, and thus a predisposition to thrombotic events such as cerebrovascular accidents. This need for precise regulation is all the more critical given that the RBCs have a finite lifetime of approximately 120 days, mandating the production of $2x10^9$ such cells per day.

The close relationship between oxygen delivery and RBCs needs the ability of red cells mass (RCM) to change in response to fluctuations in oxygen tension (Figure 1).

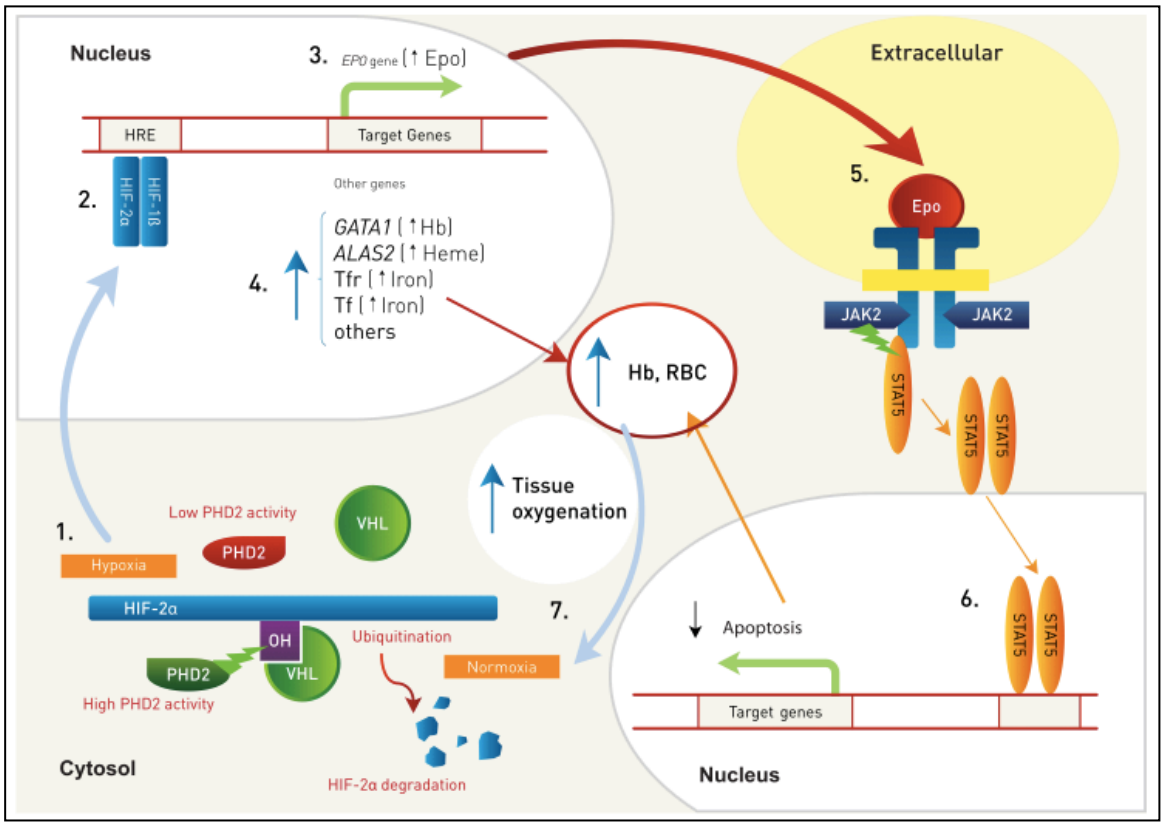

Fig. 1: The balance of RCM is regulated by the erythropoietin hormone which synthesis is under control of the OSP consequently to tissue normoxia or hypoxia conditions.

The glyco-protein hormone erythropoietin (EPO) regulates RCM responding to oxygen tension changes (1–3).

In adults, the main source of EPO is the kidney and the committed cells (Renal Epo Producing Cells, REPC) are the peritubular interstitial (4-10) found in the renal cortex (predominantly juxtamedullary region). (Figure 2).

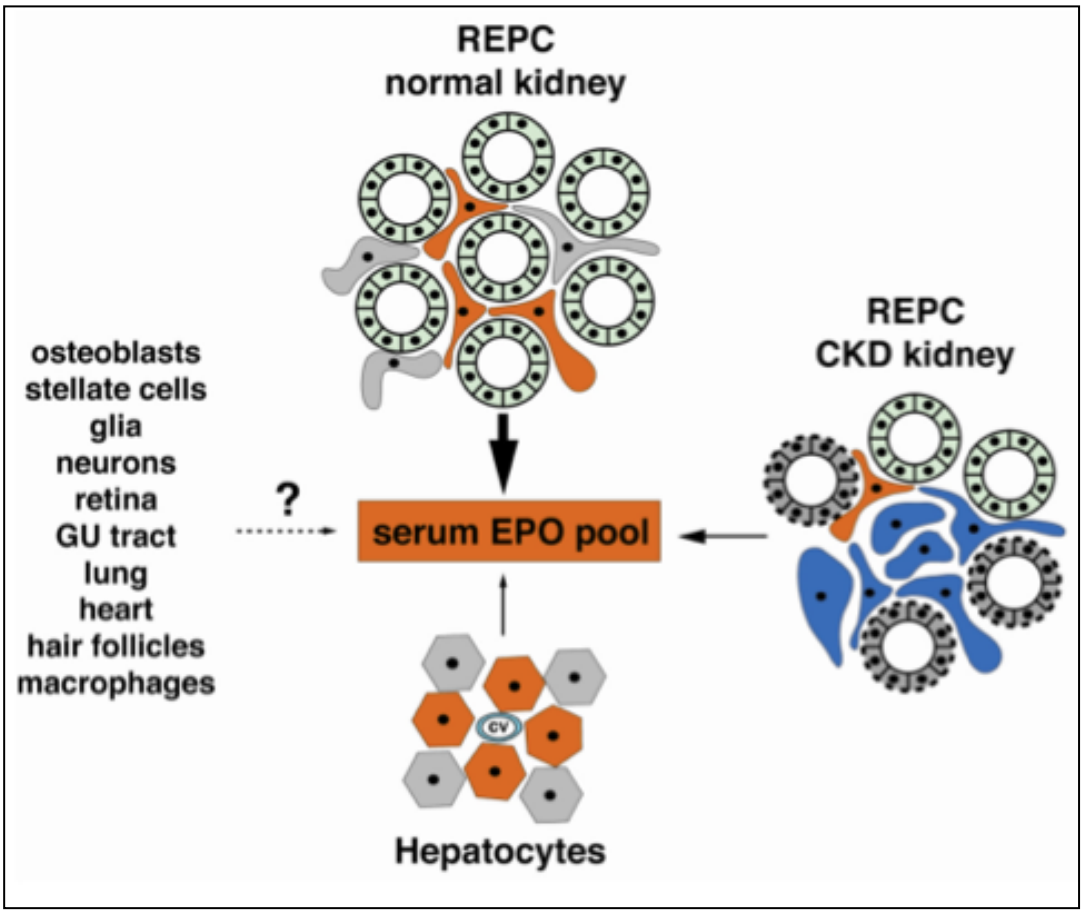

Fig. 2: Districts with EPO-producing capacity. Hepatocytes in liver and mostly the REPC in kidney are the major contributor of EPO synthesis.

Glyal cells in the central nervous system have recently been identified as another source of EPO (11).

EPOs levels in normal subjects are about 10 pg ml^{-1}. Under tissue hypoxia conditions, the *EPO* gene transcription is triggered, with a consequent increase in EPO circulating levels.

EPO binds to the EPOR, which is expressed on erythroid progenitors, and initiates an intracellular signaling cascade resulting in the recruitment and activation of Janus kinase 2 (JAK2), which, in turn, activates the STAT5 (signal transducer and activator of transcription 5) transcription factor and two other intracellular signaling pathways, the MAPK (mitogen-activated protein kinase)-ERK pathway and the PI3K (phosphoinositide 3-kinase)-AKT pathway. Collectively, these signals lead to the inhibition of apoptosis and the expansion and differentiation of erythroid precursors (12). (Figure 3).

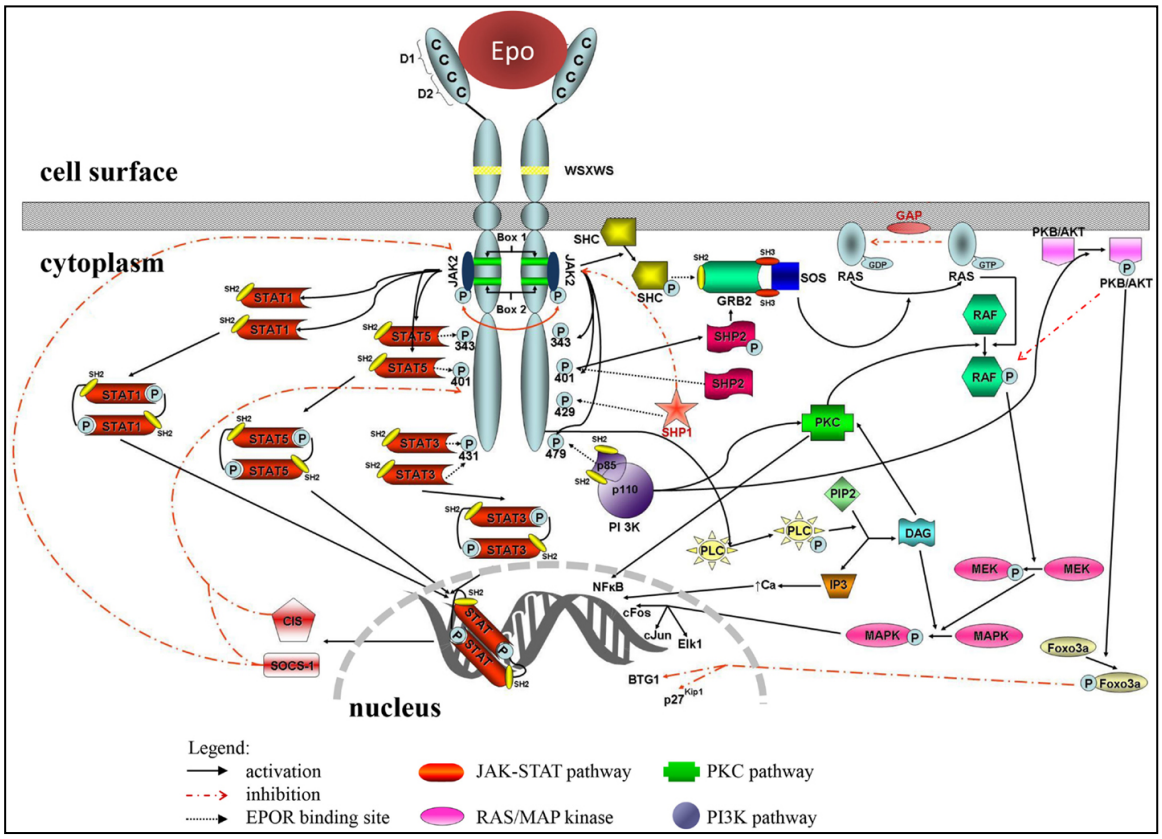

Fig. 3: EPO binds to a EPOR homo-dimer leading to activation of the EPO-EPOR signaling cascade. Main signaling pathways activated by EPO are JAK2/STAT5 pathway, PI3K pathway, RAS/MAPK pathway.

The result is an increase RCM that serves to improve oxygen delivery, and down-regulates EPO levels in a negative feedback loop to maintain RCM at an appropriate level for a given tissue oxygenation.

Erythrocytosis occurs when there is an increase in the RCM to more than 125% of the predicted value for the body mass of the patient (13). This condition usually becomes manifest with HB levels above 185 or 165 g/L (14) HCT above 51 or 48% (15), respectively in males and in females.

The HB concentration and the HCT can be influenced by the plasma volume (16). Any reduction in plasma volume may cause a relative erythrocytosis with raised HCT and HB levels. In contrast, true erythrocytosis has many causes (Table 1).

Relative		Dehydration Stress Diuretics Burns Neonatal
Absolute	Primary	Polycythemia Vera (PV) *Primary Familial and Congenital Polycythemia* (PFCP)
	Secondary	<u>Appropriate secretion of EPO</u> Altitude BPCO Cardiovascular shunt Pickwick Syndrome High affinity HB Deficiency of BPGM Methemoglobinemia Smoke
		<u>Inappropriate secretion of EPO</u> Renal Mass (cysts, hydronephrosis, cancer, transplant) *Oxygen Sensing Pathway (OSP) polycythemias* Neoplasms

Tab. 1: Classification clinical / pathological erythrocytosis (congenital forms are in *italics*)

Primary erythrocytosis is associated with low or subnormal EPOs levels and is characterized by the hyperactivity of erythroid progenitor cells without requirement for EPO. These findings are consistent with an intrinsic defect of erythroid progenitors and related to aberrant JAK2-mediated signaling. In the case of secondary erythrocytosis, the EPOs level is inappropriately high for the RCM.

The most common example of primary erythrocytosis is Polycythemia Vera (PV), which is an acquired stem cell disorder that exhibits trilineage proliferation. Most clinical symptoms in PV are related to clonal erythropoiesis, but the disorder also affects the megakaryocytic and myeloid lineages. PV is uniformly associated with acquired gain-of-function mutations in the *JAK2* gene (OMIM #147796). Almost all cases (95%) of PV present a point mutation located in exon 14 of *JAK2* that produces a V617F substitution (17–21); a small percentage (3%) of cases are associated with a

mutation involving exon 12 of *JAK2*. With both types of mutations, the resulting constitutive activation of the kinase bypasses the need for the activation of EPOR by EPO (17) and leads to the EPO-independent proliferation of erythroid precursors. The marked expansion of RCM leads to downregulation of EPO synthesis and hence low EPOs levels.

A less frequent form of primary erythrocytosis arises from mutations in the *EPOR* gene (OMIM #133171) (22). It is associated with subnormal or undetectable EPOs levels. All mutations cluster within a 180-bp region in exon 8. Most of them result in C-terminal truncations that remove a cytoplasmic domain containing binding sites for negatively regulatory factors. The mutated receptor is hypersensitive and, when stimulated by EPO, produces abnormally prolonged activation of downstream signaling pathways.

The causes of secondary erythrocytosis are heterogeneous and include chronic lung disease, right-to-left cardiopulmonary shunts, sleep apnea, high-altitude living, and less commonly, EPO-producing tumors that can be either benign or malignant. Various tumors may secrete EPO, but it occurs more commonly in renal cell carcinoma, cerebellar hemangioblastomas, and hepatocellular carcinoma. Upon removal of the tumor, the EPOs level drops, and HCT returns to normal levels (23). Secondary erythrocytoses can also be caused by molecular lesions which ultimately lead to impaired oxygen delivery. Causes in this category include high–oxygen affinity HB variants (HBB, OMIM #141900; HBA1, OMIM #141800; HBA2, OMIM #141850), deficiency of BPGM (OMIM #613896), or methemoglobinemia. A major cause of erythrocytosis related to abnormal HB function is the presence of a high–oxygen affinity HB variant. Approximately 100 HB variants have been described, and all exhibit a reduced ability to release oxygen to the tissues. Mutations that cause high–oxygen affinity variants result in structural alterations that occur at the α1β2 interface and in regions involved in stabilizing the tense state of HB (24). The resultant oxygen dissociation curve is shifted to the left, thereby depressing the p50 value, which is a measure of oxygen affinity. Most high–oxygen affinity HB variants are undetectable by routine laboratory tests and must be diagnosed by determining the oxygen-dissociation curve, an assay that is usually performed in specialized centers (24). They are often misdiagnosed. Screening of a group of idiopathic erythrocytosis (IE) patients recently detected these HB variants at a prevalence of 3% (25). High–oxygen affinity HB variants exhibit autosomal dominant transmission and are often

associated with a family history. Sequencing both the α- and β-globin genes is also helpful in diagnosing this form of erythrocytosis (25).

Deficiency of BPGM results in decreased 2,3-BPG levels, which causes the HB molecule to remain in a high-affinity state. Mutations that impair BPGM function are characterized by erythrocytosis but are very rare. They are inherited in an autosomal recessive pattern and are associated with seemingly normal EPO levels that are inappropriately high for the RCM (26). Methemoglobinemia is another cause of deregulated oxygen delivery from HB (27). During normal oxygen delivery, there is spontaneous oxidation of the ferrous iron contained in the heme moiety to the ferric state. Consequently, HB is converted to the methemoglobin form. This form is less efficient at binding oxygen, and in some individuals there is methemoglobinemia with a compensatory erythrocytosis. Methemoglobinemia can develop from three causes: a HB M variant; or deficiency of cytochrome *b*5 reductase, which converts the ferric ion to a ferrous ion, and, extremely rarely, cytochrome *b*5 deficiency (27). The HB M group of variants has structural alterations of the heme pocket due to amino acid changes that allow spontaneous oxidation of the ferrous ion. These variants are inherited in an autosomal dominant manner. The α-globin chain variants present at birth, whereas the β variants manifest postnatally as chain synthesis switches from γ- to β-globin. HB Ms are easily distinguished by routine laboratory tests, in contrast to high–oxygen affinity HB variants.

To conclude, acquired erythrocytosis usually manifest at mature ages and can be subdivided into two groups on the basis of clonality: PV is clonal, and other acquired secondary forms caused by tissue hypoxia or EPO-producing tumors are polyclonal.

In most instances erythropoiesis is polyclonal and, in familial cases it is inherited in a Mendelian manner.

Thus, absolute erythrocytosis can arise from:

- Intrinsic defects in erythroid progenitors that lead to hypersensitivity to EPO (PV and EPOR defects).
- Molecular defects that impair the ability of HB to deliver oxygen to tissues and that result in tissue hypoxia and hence activation of the EPO pathway (Pathological HB, methemoglobinemia)
- Defects in the OSP resident in the kidney and liver that transduces changes in oxygen delivery to changes in *EPO* gene transcription (OSP polycythemias);

- Physiologic or pathophysiologic changes aside from HB defects that also lead to inappropriately high levels of EPO and excessive activation of the EPO pathway (a category that also includes EPO-producing tumors)(secondary erythrocytosis);

Congenital and familial erythrocytosis can be either primary or secondary and are associated with an array of EPOs levels, which is indicative of the multiple origins of this disorder. One of the familial erythrocytosis is due to EPOR mutations, which is inherited in an autosomal dominant manner (Table 2) (28). It is designated Familial Erythrocytosis Type 1 (ECYT1, number 133100) in the Online Mendelian Inheritance in Man (OMIM) (http://www.ncbi.nlm.nih.gov/sites/entrez? db=omim). The other three recognized forms of familial erythrocytosis, ECYT2-4, are secondary erythrocytoses that derive from a deregulated EPO production due to mutations in the OSP.
CE are the focus of the remainder of this thesis.

Name	**OMIM**	**Gene**	**Inheritance**	**EPO**	**Clinical Feature(s)**
ECYT1	133100	*EPOR*	Autosomal dominant	Low	-
ECYT2	263400	*VHL*	Autosomal recessive	High	Stroke, hemangiomas, pulmonary hypertension
ECYT3	609820	*PHD2*	Autosomal dominant	Normal	Paraganglioma
ECYT4	611783	*HIF2α*	Autosomal dominant	High	Pulmonary hypertension, thromboses

Tab. 2: NCBI classification of CE (HB and BPGM forms not shown).

CE may be characterized by high levels of EPOs or inappropriately high. These Secondary Erythrocytosis result from mutations in genes that encode proteins embroiled in OSP. It has been documented gene mutations in hypoxia inducible factor

2alpha (*HIF2α*), the prolyl-hydroxylase 2 (*PHD2*) and the tumor-suppressor Von Hipple-Lindau (*VHL*) (29).

EPO production grows in conditions of hypoxia or anemia due to reduced oxygen tension. In fact, under normoxia, hydroxylation of HIF2*α* subunits by prolyl-hydroxylases is required for binding to the pVHL–E3-ubiquitin ligase complex. After polyubiquitination, HIF2*α* is degraded by the proteasome. During hypoxia, HIF2*α* subunit is not degraded and translocates to the nucleus where it binds to the HIF-β subunit. HIF-α/β heterodimers then bind to HIF-DNA consensus-binding sites resulting in increased transcription of more the 200 HIF-target genes (Figure 4), for example, *EPO, VEGF*, and *glucose transporter-1*. HIF2α is the main isoform of HIF and it's involved in the regulation of *EPO* transcription. Furthermore it also regulates other genes involved in cell survival under conditions of low oxygen tension as the gene of heme-synthase (*ALAS2*), the production of globin chains (*GATA1*) and iron regulation (*TRF2, TF*) (30-34).

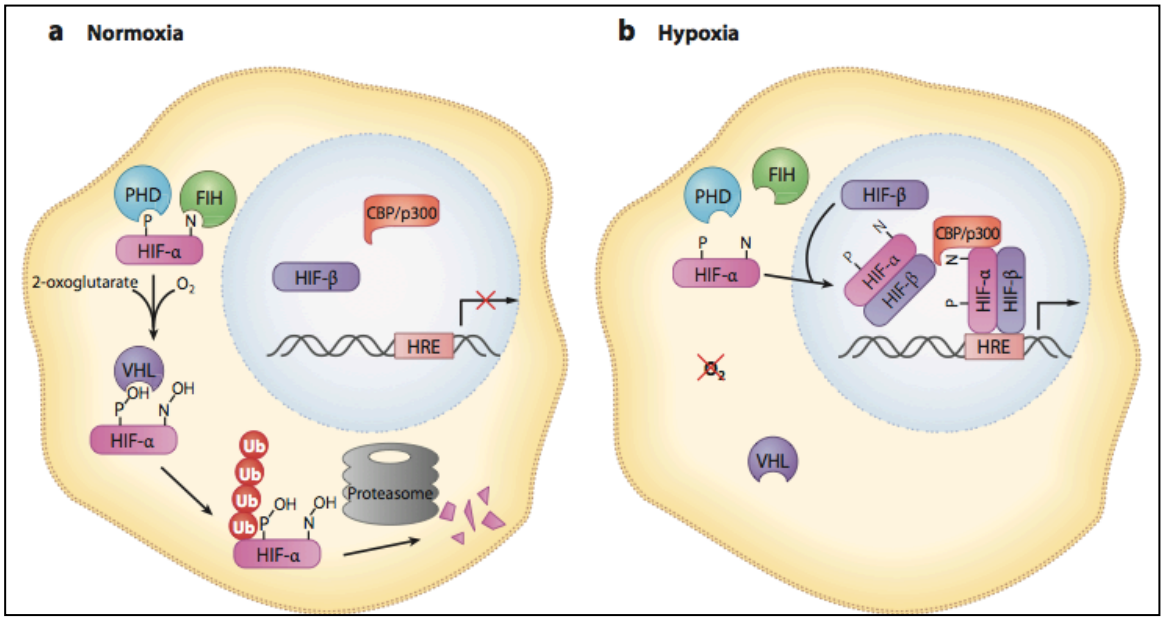

Fig. 4: Representation of the proteins involved in the OSP and their functional mechanism in base on tissue oxygenation conditions.

The *VHL* gene (OMIM #608537) maps on the short arm of the third chromosome (locus 3p25.3) and spans 10 kb. The *VHL* gene encodes a 4.7 kb mRNA translated from two translational initiation sites (+1 and +54). These 2 protein products are a 30-kD full-length form (p30) formed by 213 amino acids and a 19-kD smaller form (p19) formed by 160 residues, both are functionally active (35). pVHL is a

component of the protein complex that includes elongin B, elongin C, and cullin-2, and possesses ubiquitin ligase E3 activity (Figure 5).

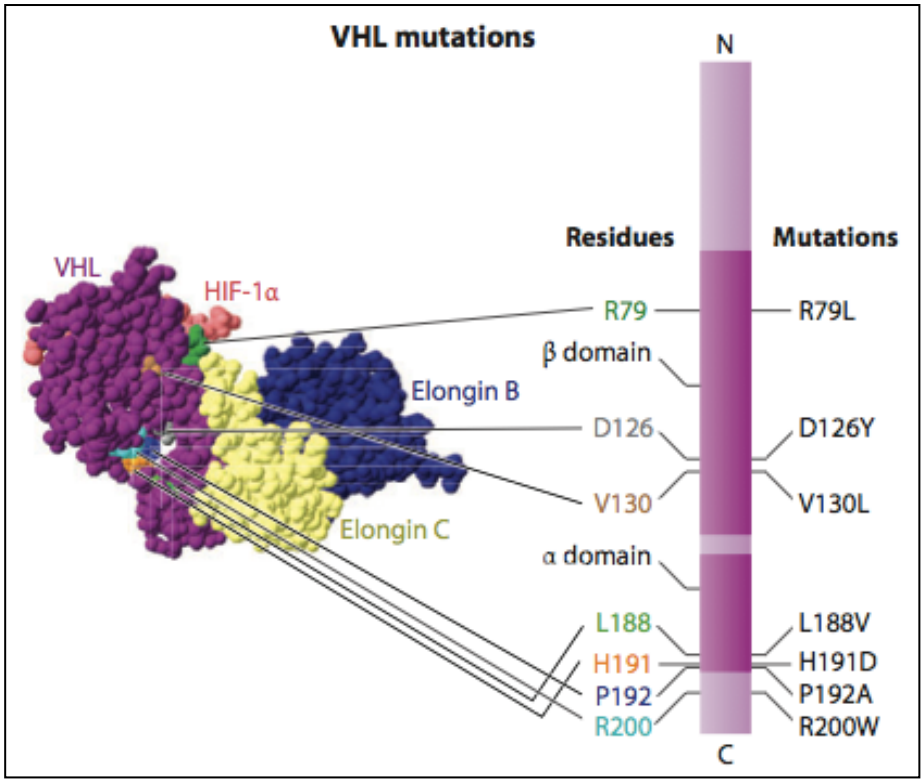

Fig. 5: Structural representation of the protein encoded by *VHL* gene.

More than 400 germline mutations in the *VHL* gene have been described as associated with the VHL disease (OMIM #193300) (36).

VHL syndrome is a dominantly inherited familial cancer syndrome predisposing to a variety of malignant and benign tumors but VHL disease is outside the scope of this thesis.

The first loss-of-function mutation in the *VHL* gene associated with CE was first described in more than 100 individuals from about 80 families living in the mid-Volga River region of European Russia named Chuvashia. There erythrocytosis is endemic, whereas its worldwide frequency is very low. Chuvash polycythemia derives from a homozygous variant c.598C>T (p.Arg200Trp) in *VHL* gene (37). Also in Italy on the island of Ischia outside the bay Naples, and in non-Chuvash had been observed the *VHL* c.598C>T homozygosity mutation. Both non-Chuvash and Italian patients had the same haplotype as the Chuvash cohort, suggesting a common ancestor, which suggested this mutation may be endemic in other parts of the world (38, 39). Sixteen additional *VHL* variants associated with CE have also been

described. Four of them presented in the homozygous state, whereas the other cases were either compound heterozygosity or heterozygotes. *VHL* is associated with erythrocytosis (CE type 2; MIM #263400; Table 2) it is considered a recessive disease.

PHD2 is an enzyme encoded by the *EGLN1* gene (OMIM #606425), which is located on chromosome 1q42.1, and it is comprised of five exons (Figure 6). There are three PHD isoformes (PHD1, PHD2, and PHD3), but PHD2 is the primary functional isoform that catalyzes the prolyl hydroxylation of HIF-α, using oxygen as a cosubstrate (40, 41).

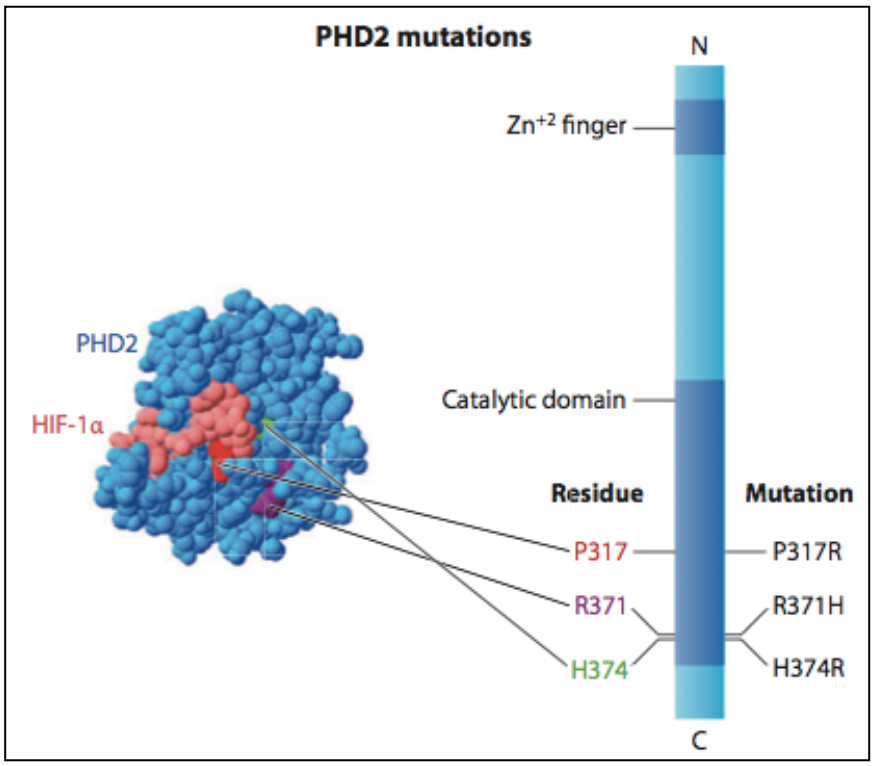

Fig. 6: PHD2 domains and its tridimensional structure

CE type 3 (OMIM #609820) is characterized by loss-of-function mutations of *PHD2* (Table 2) with autosomal-dominant inheritance.

The c.950C>G, p.Pro317Arg was the first mutation described (41) in three members of two generations of a family. This mutation resulted in a substitution in a highly conserved region of the protein. *In vitro* functional expression studies show that the mutant protein had significantly decreased enzyme activity. In the son and daughter of the propositus, the HCT was elevated despite inappropriately normal EPOs levels,

suggesting deregulated EPO production.

Since then, more than 22 patients were found to carry 16 mutations in PHD2 gene, all of them heterozygous, and the mutations include: 12 missense, two nonsense, one small duplication, and one small deletion. Among missense mutations, the c.471G>C (p.Gln157His) was found to coexist with the JAK2 p.Val617Phe somatic mutation, the latter probably being the primary cause of the disorder. Meanwhile, the c.471G>C mutation has been categorized as a SNP (rs61750991) with a frequency of around 2% in the normal population although some studies refer to a higher frequency (42, 43). Interestingly, one particular mutation (p.His374Arg) has been described in a patient with an erythrocytosis associated with a recurrent paraganglioma (44).

The *HIF2α* gene (MIM #603349), is located on chromosome 2p21, it has 16 exons and spans at least 120 kb.

At least three transcript variants encoding three different isoforms have been found for this gene, HIF1α, HIF2α, and HIF3α. HIF1α was first identified as a mediator of EPO induction in response to hypoxia in vitro (45), however HIF2α was later confirmed to have the major role to induce EPO expression (46-49). The degradation of HIF2α occurs via the hydroxylation of the Proline residues in position 405 and 531 becoming then a target for the VHL complex.

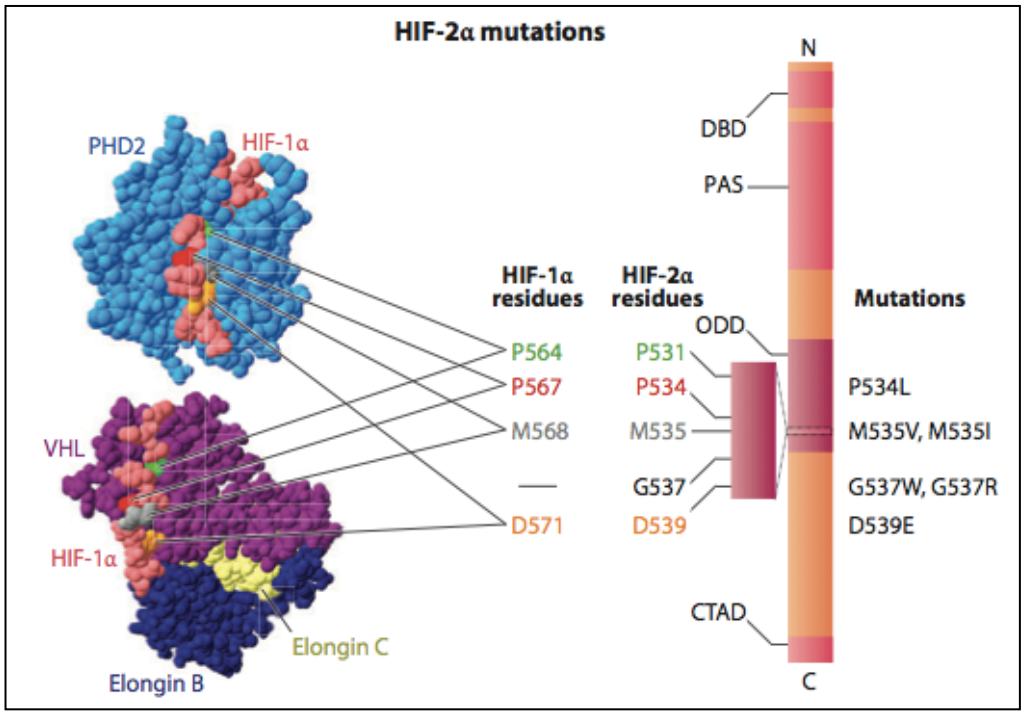

Fig. 7: Domains and several crucial residues of HIF2α

Mutations in exon 12 of *HIF2α* are responsible of familial erythrocytosis type 4 (OMIM #611783) with autosomal-dominant inheritance. At present, in 22 patients (eight sporadic cases and four families) heterozygous *HIF2α* mutations have been reported. p.Gly537Trp, p.Gly537Arg, p.Met535Val, and p.Pro534Leu (49-51) are the first missense *HIF2α* mutations found in erythrocytosis patients. The group of Martini in 2008 (52) described another pathogenic mutation, p.Met535Ile, and more recently, three additional missense mutations have been described, p.Asp539Glu, p.Met535Thr, p.Phe540Leu (53, 54). Whereas in 2012 the group of Lorenzo (55) identified a germline heterozygous missense mutation c.1121T>A (p.Phe374Tyr) in exon 9 in a polycythemic patient who developed pheochromocytoma/paraganglioma.

Overall, more than 160 known mutations are associated with CE and the majority of them (about 100) are in globines. However, more than 70% of cases of erythrocytosis the molecular cause is not recognized. Therefore, we refer to these cases as IE.

Among CE, PFCP is characterized by reduced or inappropriately low level of EPOs due to EPOR germline mutations causing a gain-of-function of the receptor. (56, 57).
The coding region of the *EPOR* gene is contained within 8 exons spanning approximately 6 kb. Its cytogenetic location is 19p13.2, which is the short arm of chromosome 19 at position 13.2. The primary transcript 2,056 bp long encodes the EPOR which is a member of the cytokine receptor family, a protein of 508 amino acids (MW 66 kDa) (figure 8).
Alternatively spliced forms of the EPOR have been identified, one of which has a truncated cytoplasmic domain. The shortened transcript is expressed at high levels in immature erythroid progenitor cells. In contrast, the expression of the full-length receptor increases as progenitor cells mature (58).
De la Chapelle et al. in 1993 (28) discovered the first mutation in the *EPOR* gene in a successful Finnish sportsman and others 29 family members. Subsequently, more than 26 heterozygous variants have been found in patients with CE. All of these mutations are located in exon 8, which encodes the C-terminal negative regulatory domain of the protein. In total, 19 are *frameshift* or *nonsense* mutations leading to cytoplasmic truncation of the receptor and loss of the C-terminal negative regulatory domain (Figure 8). All these 19 mutations induce a gain-of-function of the receptor and are

associated with PFCP. Of the remaining variants, six are missense mutations (c.1138C>G, c.1310G>A, c.1462C>T, c.1460A>G, c.1362C>G, c.1445G>A) (59, 60).

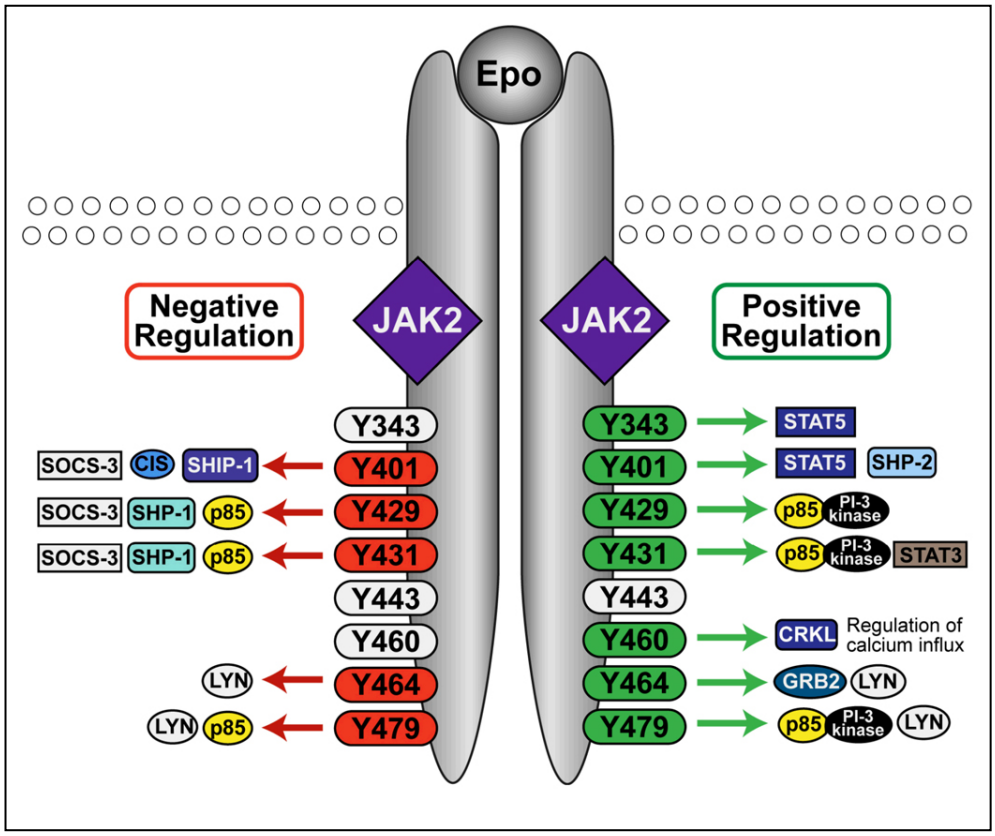

Fig. 8: 3D representation of EPOR with all cytoplasmic residues responsible for the receptor activation or inhibition.

In spite of its cause, high numbers of RBCs and high levels of HCT resulting in hyperviscosity may cause signs and symptoms ranging from headaches, dizziness, to epistaxis, dyspnea on exertion. Thrombotic and hemorrhagic events with an increased morbidity and mortality have also been described. Clinical symptoms can be effectively reduced by bloodletting, but the recovery of normal HCT is not necessarily reduce cardiovascular complications (61).

Among CE, the cohort of Chuvash patients is today the most widely studied. The homozygotes cases had a lower survival compared to heterozygous subjects mainly due to a high incidence of both arterial and venous thrombosis (62),

There is little information regarding the clinic of patients with *PHD2* mutations. Thromboembolic events have been reported (63, 64) even at a young age and overall it seems that the clinical risk of these patients is similar to those with Chuvash erythrocytosis (65).

Paragangliomas seem to be associated with mutations of *HIF2α* (66).

Yet little is known about the clinical of erythrocytosis due to alterations of EPOR.

In conclusion, high HCT and increased viscosity can cause various complications, including thromboembolic events also in young age, but in the absence of clinical and biomolecular data currently not available, it is not possible to draw a clear picture of these patients and even less, to determine what is their best management.

In the present study, we report our experience on a large cohort of sporadic adult patients with high HCT, not affected by PV or acquired secondary erythrocytosis and we describe the functional activity of 1 PHD2 and 2 EPOR genes novel mutations.

MATERIALS AND METHODS

PATIENTS

Among patients referred to our surgery with high HB and/or HCT levels (male: HB > 185 g/L, HCT > 52%, females: HB > 165 g/L and HCT > 49%) we selected sporadic adult cases in whom an absolute erythrocytosis has been diagnosed, but PV and secondary acquired erythrocytosis were excluded. Therefore, we collected a careful medical history paying particular attention to the familial history with the main aim to identify other cases of Erythrocytosis in the kindred. We also registered the patients' clinical symptoms giving particular attention to: headache, dizziness, paresthesia, aquagenic pruritus, previous haemorrhages or thrombosis. A careful clinical examination was carried out mainly for reveal hepatosplenomegaly, angiomas, hemangiomas and altered cardiovascular function.

In all patients, arterial blood gas analysis, abdominal ultrasonography to evaluate liver, spleen and kidneys, had been performed. Echocardiography and Doppler ultrasound had been performed if appropriate.

When secondary erythrocytosis (cardiac, pulmonary, renal) had been ruled out, we evaluated EPOs (EIA method) and we searched *JAK2* mutations (both V617F and exon12 mutations) to exclude PV. P50 evaluation was carried out with Rapid Point 405 Analyzer (Siemens) to identify high oxygen hemoglobinopathies. When found P50 high values, the germline and somatic DNA samples had been sent to specialized centers for the study of hereditary hemoglobinopathy. Thereafter, if erythrocytosis is considered secondary/reactive we excluded the patients from the study.

RBC-Granulocytes isolation was performed on patients' venous peripheral blood samples (each 5-10 ml total) collected in four 3mL EDTA tubes each. Complete blood counts were carried out with Sysmex XP300 cell counter to ensure the samples had cell counts within the reference range.

We executed a Histopaque-1077 (Sigma Aldrich, St. Louis, MO USA) density gradient separation (Figure 9) following the protocol below:

1. Pipet 5ml Histopaque-1077 in 15ml conical centrifuge tube
2. Carefully layer 5ml EDTA blood atop the Histopaque-1077 layer
3. Centrifuge at 400 x g for 30 min at room temperature
4. Aspirate RBC - Granulocytes (bottom layer) and transfer to 1,5ml Eppendorf tube

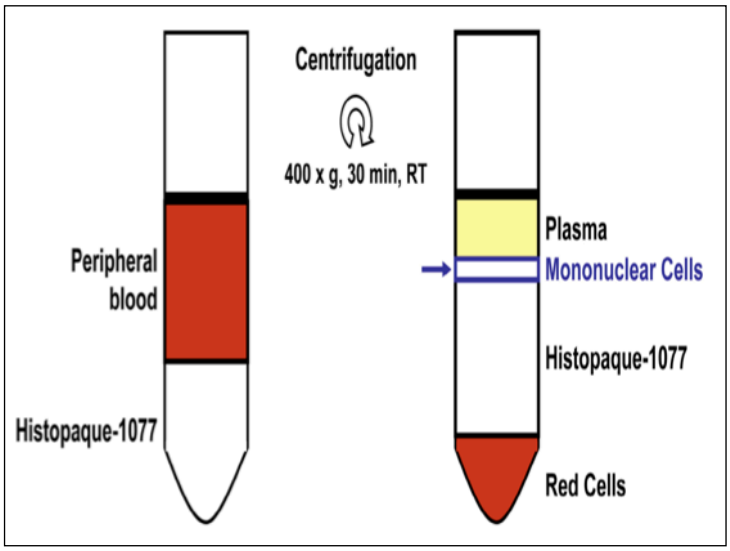

Fig. 9: Blood separation in Histopaque-1077 density gradient.

The genomic DNA was then extracted with automatic extractor Maxwell 16 System (Promega) (Figure 10) that uses a paramagnetic-particle handling system that processes samples using Maxwell reagent kits, providing consistent yield and purity. The kit is optimized to process a wide range of volumes of fresh or stored human blood samples. Up to 400µL of blood sample can be processed to yield up to 15µg of DNA.

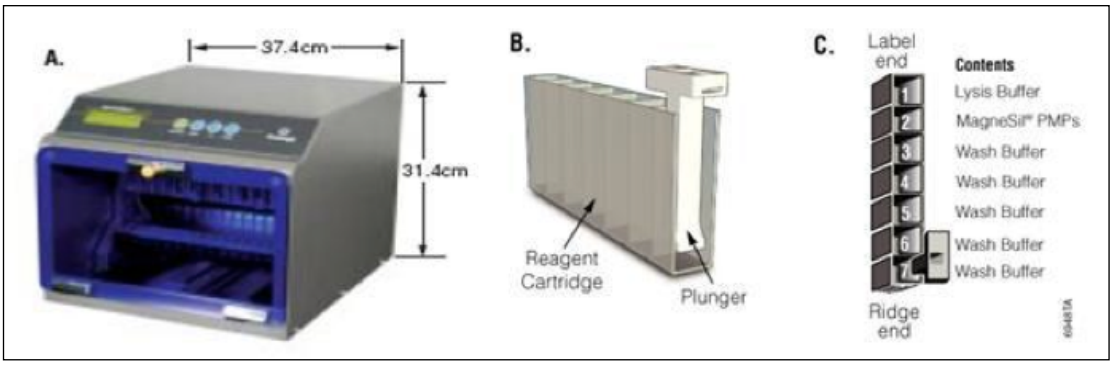

Fig. 10: Maxwell 16 System automatic extractor (Promega).

The presence of JAK2 V617F mutation was examined with Polymerase Chain Reaction (PCR) analysis and allele-specific quantitative PCR (qPCR) for the definition of the allelic burden if present. *JAK2* exon 12 mutations were searched by Sanger sequencing.

The germline DNA was collected by brushing the oral mucosa using Charge Switch gDNA Buccal Cell Kit (Invitrogen) to check the presence of mutation in other cell lines if found.

In cases of low EPOs values we proceed in the first instance to search for mutations in *EPOR*, whereas when EPOs was elevated or inappropriately high we proceed to analyze *VHL, PHD2 and HIF2α* genes. (Figure 11).

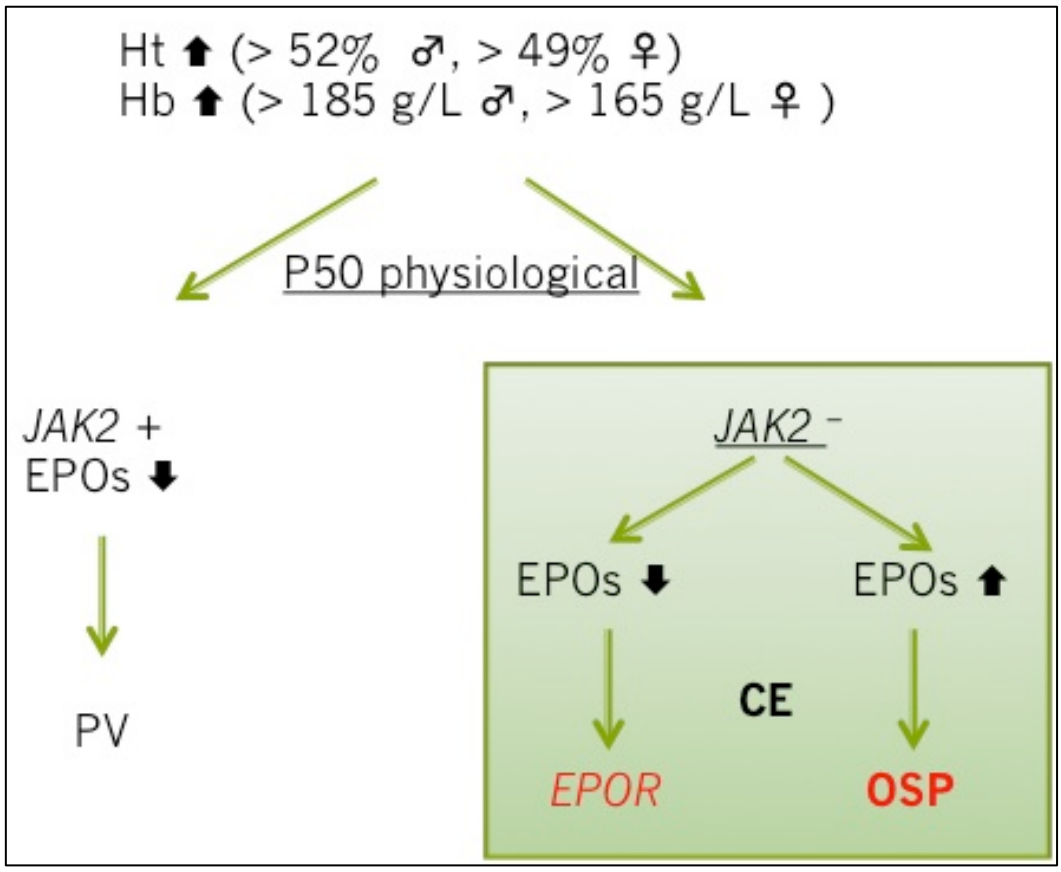

Fig. 11: Flowchart genetic investigations EI.

For selected patients we drew a genetic profile regarding the molecular investigation of *EPOR, PHD2, VHL or HIF2α genes*.

The amplified genomic areas in exam were obtained by PCR analysis. We designed all primers using Primer 3 software (http://frodo.wi.mit.edu/primer3) (Table 3).

PRIMERS	EXON	ANNEALING	CYCLES
EPOR F 5' – gcctctatgactgggagtgg – 3' EPOR R 5' – ctgagagaggcctcgccat – 3'	8	60°C for 1'30"	40
HIF2A F 5' – tgacacagccaagtctgagg – 3' HIF2A R 5' – gtatcagatggctggggaga – 3'	12	60°C for 1'30"	40
VHL 1F 5' – agcgcgttccatcctcta – 3' VHL 1R 5' – gcttcagaccgtgctatcgt – 3'	1	60°C for 50"	40
VHL 2F 5' – ctcccaaagtgctgggatta – 3' VHL 2R 5' – tggataacgtgcctgacatc – 3'	2	60°C for 50"	40
VHL 3F 5' – gcaaagcctcttgttcgttc – 3' VHL 3R 5' – caaaaatgccaccaccttct – 3'	3	60°C for 50"	40
PHD2 1AF 5' – gcacaggccctattctctca – 3' PHD2 1AR 5' – ggcctttacttttcccttgg – 3'	1	60°C for 40"	35
PHD2 1BF 5' – ggccaagggaaaagtaaagg –3' PHD2 1BR 5' –gaagtcgtccaccacacag– 3'	1	60°C for 40"	35
PHD2 1CF 5' – tctgtgtggtggacgacttc – 3' PHD2 1CR 5' – ccttacggggagctacacaa – 3'	1	60°C for 40"	35
PHD2 2F 5' – tcttgtgttccctataaatgttaatgt -3' PHD2 2R 5' – tccactcctaatacctgagactga –3'	2	60°C for 40"	35
PHD2 3F 5' – ttgtccttgcatcagtgccat – 3' PHD2 3R 5' – cagattccctcctgtcctacc – 3'	3	60°C for 40"	35

Tab. 3: Primers sequences for PCR analysis. In the table are also reported annealing temperatures and cycles of all PCR we tuned.

The amplification by PCR was performed, in a volume of 12.5 µl, according to the protocol given in table 4, using GoTaq® Colorless Master Mix (Promega) All annealing temperatures and reaction cycles are described in table 3.

	Quantity µl
GoTaq® Colorless Master Mix	6,25
Primers Forward (100ng/µl) 10µM (0,3 µM final)	1
Primers Reverse (100ng/µl) 10µM (0,3 µM final)	1
H_20 Pyrograd	2,75
DNA	1,5

Tab. 4: PCR reaction mix.

We evaluated the success of the reaction with electrophoresis on 1,5% agarose gel TBE 1X colored with GelRed (Biotium), loading 5 µl of amplification product.
The amplification product had been then purified with ExoSAP-IT (Affymetrix) in order to remove primer excess, the single-stranded DNA fragments and the free dNTPs in solution.
To 1µl of PCR product it was added 1µl of enzyme; then mixture was incubated in a thermocycler (37° C for 15 minutes, then at 80° C for 15 minutes for enzyme inactivation).
After the purification we performed the sequencing reaction using an automatic sequencer ABI-PRISM 3100 Genetic Analyzer (Applied Biosystem).
The sequencing reaction was performed from 100-500 ng of nucleic acid according to the protocol given in the BigDye Terminator v1.1 kit (Applied Biosystems) (Table 5).
The thermal profile of the reaction is described in table 6 .

Reaction mix	**Vol (µl)**
Purified DNA	100-500 ng
primer Forward/Reverse (10 µM)	1
BigDye 1.1	2
H_2O Pyrograd	3

Tab. 5: Sequencing reaction mix.

T (°C)	**Time**	**cycles**
96	10"	30
60	5"	
60	4'	

Tab. 6: Sequencing reaction thermal profile.

Then we purified the obtained amplification products with GE Healthcare columns (Buckinghamshire); 4 µL of amplification product were mixed with 16 µL of formamide and loaded into the automated sequencer.
The electropherograms were analyzed with the software Chromas (Winlmp Self-Extractor Copyright 2000 Technelysium Pty Ltd) and sample sequences alignment with the wild type genes sequences was performed using BLAST software (http: //www.ncbi. nih.gov/BLAST).

FUNCTIONAL STUDY OF *PHD2* MUTATION

To evaluate the *PHD2* mutation function, in cooperation with the group led by Prof. Sylvie Hermouet University of Nantes, France) we proceeded with the following 4 steps:

1. Computational study;
2. Construction of mutant plasmids;
3. *In cellulo* luciferase reporter assay based on hypoxia response elements (HRE) reporter gene to test the HIF transcriptional activity;
4. *In vitro* hydroxylation test to assess the hydroxylation capacity of PHD2 variants;

IN SILICO STUDY

Conservation of sequences and residues can provide information on functional significance.

The mutation identified by nucleotide sequencing had been submitted to a preventive computational study in prediction sites using three software: POLYPHEN-2 of Harvard (67), SIFT of the Craing J. Venter Institute (68), and MUTATION TASTER by Team Charitè (69). The resulting data defined whether the nucleotide variation alters or not the protein.

We studied also the conservation of the mutated residue in different animal kingdoms to provide information on its functional significance. To assess the conservation of the mutated residue we generated a multiple sequence alignment covering a huge range of taxa from different phyla. Using the MULTIZ whole genome multiple alignment algorithm (70), implemented in the University of California, Santa Cruz, Genome Browser (71), we extracted the vertebrate homologs across 100 species including primates, mammals, birds and fish, while we obtained the invertebrate homologs with FlyBase implemented InParanoid algorithm (72).

CREATION OF MUTANT EXPRESSION VECTORS

As already discussed above we created primers (Table 7), bearing the mutation in exam, to be used in site directed mutagenesis kit.

primer Forward	CGAATTTTTCCAGAAAGCAAAGCCCAGTTTGC
primer Reverse	GCAAACTGGGCTTTGCTTTCTGGAAAAATTCG

Tab. 7:PHD2 primers designed for the Site Directed Mutagenesis kit.

Amplification by PCR was performed, in a volume of 50 µl, according to the protocol given in table 8., using Phusion Hot Start II HF DNA polymerase (Life Technologies) following temperature cycles described in table 9.

	Quantity (µl)
5X GC rich reaction buffer	10
Plasmide (50ng/µl)	1
Primers (100ng/µl) 10µM (0,3 µM final)	1,5
DMSO	1
DNTPs 50mM	1
PHUSION Hot Start II HF Dna polymerase	0,5 (2,5U/µl)
H20 (qsp 50µl)	33,5

Tab. 8: Site Directed Mutagenesis amplification reaction mix

Temperature	Time	Cycle
98°C	1min	1
98°C	10s	6
55°C	1min	
72°C (30''/kb)	4min	
98°C	10s	20
65°C	1min	
72°C (30''/kb)	4min	
72°C	10min	1

Tab. 9: Thermal profile of the mutagenesis reaction

Once completed, the PCR reaction was cooled in ice for 2 minutes.

To digest the plasmid used as a mold (without mutation) we added 1µl of DpnI restriction enzyme specific for methylated DNA (Thermo Scientific, 20 U/µl). The digestion reaction takes place at 37° C and lasts an hour. The yield of the reaction was evaluated with electrophoresis on agarose gel 1% stained with Gel Red (Biotium).

The obtained constructs were then cloned into ultracompetent BL21 bacterial cells (NEB) according to the protocol in table 10 and the isolated clones were amplified in 5ml miniculture of LB (Luria Bertani) medium containing ampicillin at 0.1%. It was then extracted with miniprep plasmid DNA kit (Macherey Nagel) and sequenced to verify the goodness of site directed mutagenesis (Figure 12).

1	Add 1µl mut. react. in 50µl of BL21
2	30' in ICE
3	Heat-shock at 42°C for 30''
4	2' in ICE
5	Add 250µl of SOC at 37°C (Invitrogen)
6	60 min incubation at 37°C at 225 rpm
7	Seed on LB agar with antibiotics
8	Incubation at 37°C overnight

Tab. 10: BL21 transformation protocol

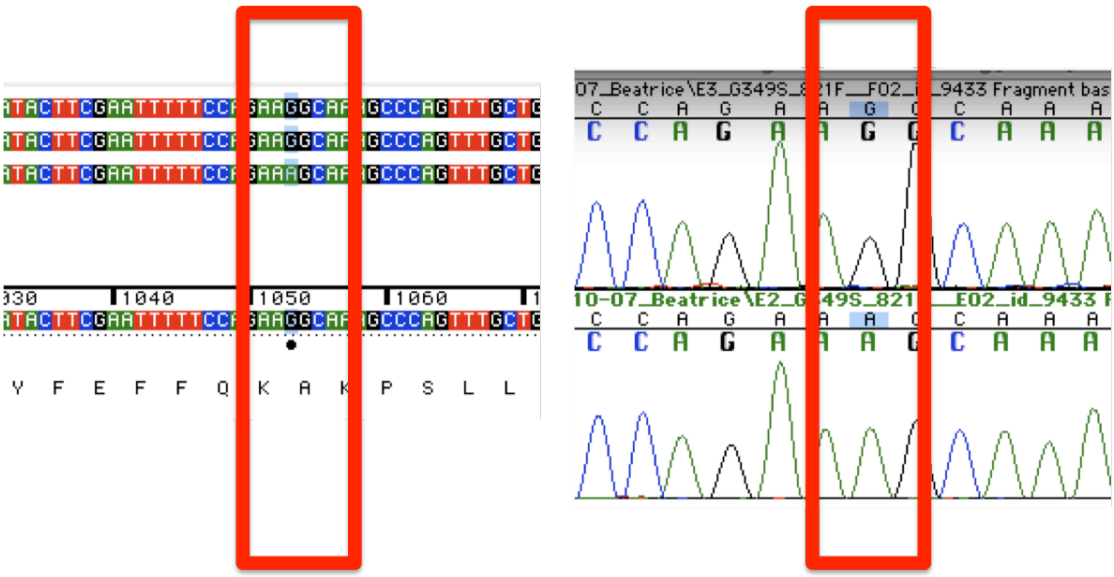

Fig. 12: Sequencing of PHD2 mutant plasmid

Once assessed the success of site-specific mutagenesis we amplified the bacterial colony containing the mutation first in a 20mL midicolture of LB medium 0.1%

ampicillin and then in 500mL maxicoluture of LB medium 0.1% ampicillin incubating overnight at 37 ° C in 225 rpm shaking.
The following day the plasmid DNA was extracted with maxiprep kit (Macherey Nagel).

In order to test the HIF transcriptional activity, accumulated after 4-6 hours under hypoxic conditions (1% O_2) in HEK293T cells, we developed a Dual Luciferase Reporter (DLR) Assay (Promega) (44). We used a pGL3 vector that expresses the firefly luciferase under the control of the HRE promoter activated by HIF.
Genetic reporter systems are widely used to study the physiology and gene expression in eukaryotic cells and the DLR Assay allows to increase the experimental accuracy (73). The term "dual luciferase" in fact refers to the simultaneous expression and measurement of two individual reporter enzymes in the same system: the firefly luciferase derived from Phatinus pyralis (Fluc) is used as an experimental reporter, and Renilla luciferase as internal control which the constitutive expression is given by cytomegalovirus promoter (CMV).
In this assay the activity of both luciferase are measured in a sequential manner from the same cell extract. It is based on the substrate specificity: the Fluc activity is measured by adding Luciferase Reporter Assay II (LAR II) and, after quenching with the addition of Stop & GLO reagent, is detected the activity of Renilla luciferase. The Fluc activity is then normalized by the activity of Renilla in order to minimize any variable, such as differences in the number of viable cells, the efficiency of transfection and lysis, which could undermine the experimental accuracy (Figure 13).

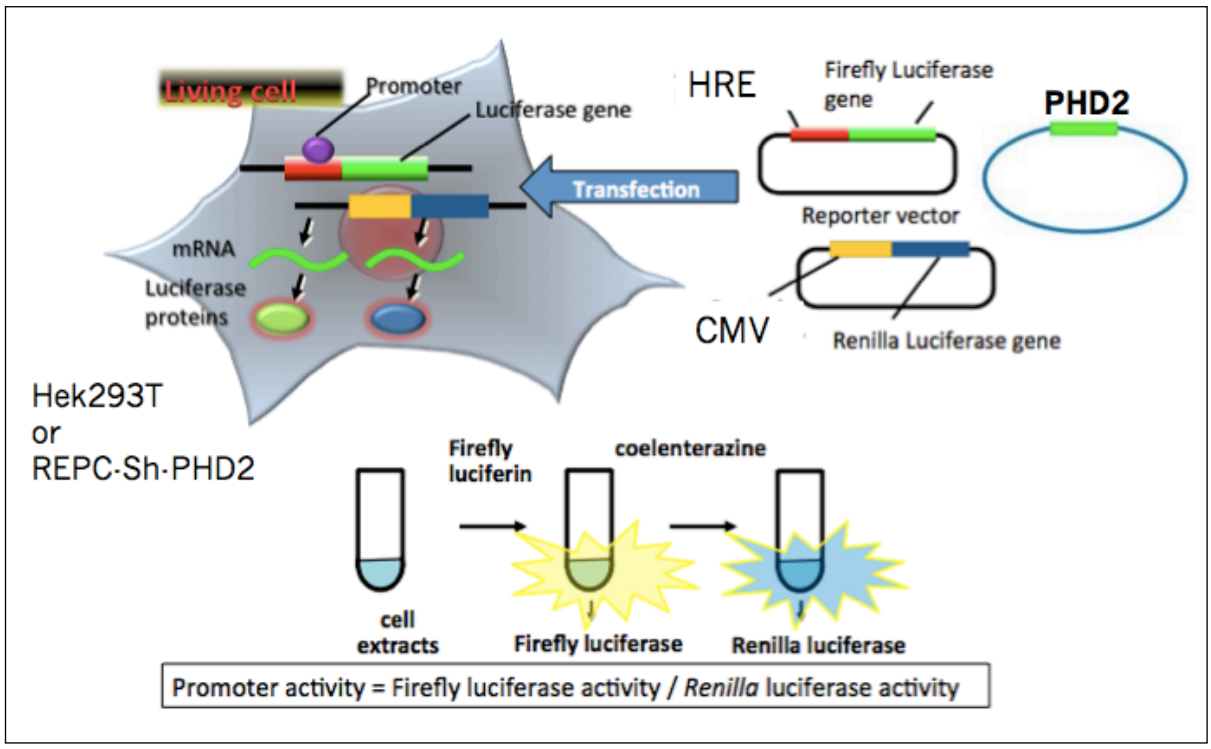

Fig. 13: Dual Luciferase Reporter Assay

The functional consequences of the mutation in the activity of PHD2 was then assessed by a hypoxia reporter assay detecting the transcriptional activity of endogenous HIF accumulated in hypoxic conditions.

We performed the DLR assay in HEK293T cells, grown in DMEM-Low glucose supplemented with 10% FBS, Pen-Strep and L-Gly. We seeded $2x10^5$ cells per well in 12-well plates 24 hours before transfection. For the transient transfection was used Lipofectamine 2000 (Invitrogen).

The cells were then transfected with 100ng of vector containing Fluc under control of HRE, 20ng of Renilla and a range of amounts of PHD2-WT or mutated plasmid.

The enzymatic activity of Fluc was measured 24 hours after transfection in both normoxic or hypoxic conditions. Furthermore we also evaluated the amount of PHD2 cells production with immunoblot analysis using mouse monoclonal antibodies anti-HA (Santacruz).

In order to minimize the noise given by endogenous PHD2, we performed the same experiment in REPC cells silenced for *PHD2*.

PHD2 is a member of the Fe(II) and 2-OG-dependent oxygenase superfamily in which almost all members follow the same overall reaction mechanism: the substrate (HIF-α) is hydroxylated in the presence of co-substrates (2-OG, dioxygen) and

cofactors (Fe(II) and ascorbic acid). The reaction is accompanied by oxidative decarboxylation of 2-OG giving CO_2 and succinate (Figure 14)

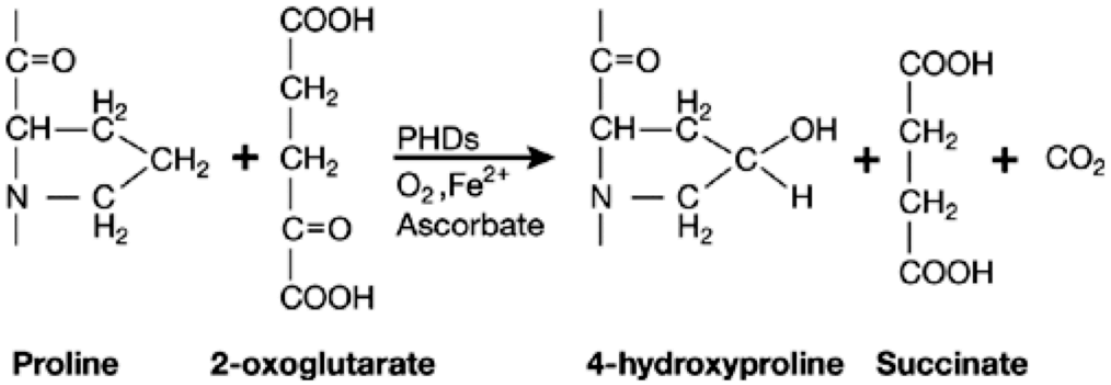

Fig. 14: Hydroxylation reaction of PHD2 enzyme with all cofactors.

To evaluate the ability of mutant PHD2 to hydroxylate HIF2α we tuned an *in vitro* assay. This reaction uses recombinant proteins produced *in vitro* with wheat germ extract to avoid the deriving contamination from endogenous bacteria hydroxylases (TNT Coupled Transcription/Translation System Promega).

The plasmid vectors contain the proline of C-terminal ODD domain of HIF1α (amino acids 556-574 or 549-575) and the *PHD2* WT or mutated.

Primarly we used PvuI (Takara) restriction enzyme to linearize plasmid vectors containing HIF-ODD, *PHD2* WT and mutated *PHD2*.

With the TnT Coupled Wheat Germ Extract Systems (Promega) we obtained all necessary proteins for the hydroxylation assay.

This *in vitro* system allows to have, in a single test tube, high yields of protein production to study bypassing all typical problems of standard methods such as long working times and transcription/translation problems (Figure 15).

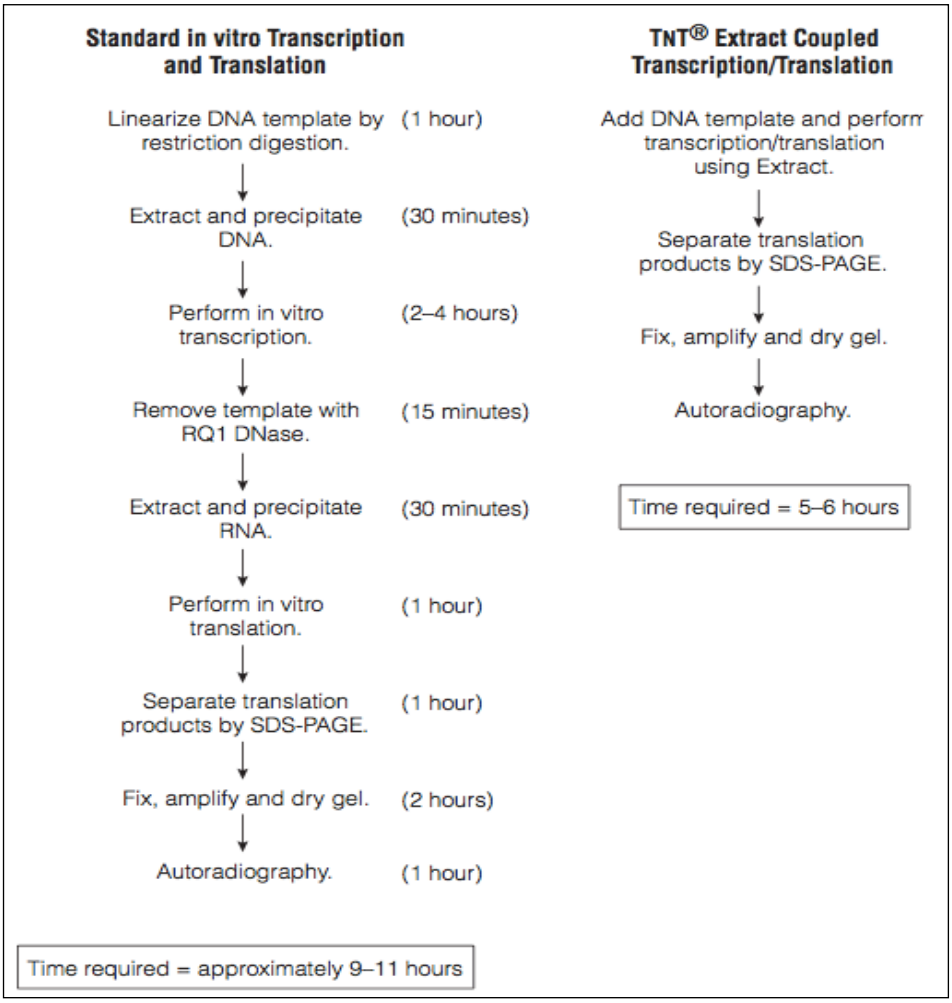

Fig. 15: TnT Coupled Wheat Germ Extract Systems (Promega)

After verified the quality of the transcription-translation reaction by immunoblot analysis using mouse monoclonal antibodies anti-HA, we continued the hydroxylation reaction: in several test tubes we mixed PHD2 WT, or mutated, with HIF1α-ODD with all cofactors necessary for the hydroxylation reaction (Fe2 +, ascorbic acid, 2-oxoglutarate) (Figure 14) (74).

The hydroxylation reaction was performed at 30°C following a time course at 0, 10, 30 and 60 minutes.

Then we compared the hydroxylation ability of the mutant PHD2 to the WT. Thus we performed an immunoblot analysis using rabbit monoclonal antibodies specific for HIF-OH (Cell Signaling).

Hydroxylation deficiency of the mutated PHD2 results in the absence of HIF-OH in the blot.

FUNCTIONAL STUDY OF TWO *EPOR* VARIANTS

For this study we proceeded with the following steps:

1. Computational study
2. Construction of mutant plasmids
3. Transfection
4. Time course

We performed a study *in silico* as already written above to understand if the mutations could impair the regular functionality of the receptor.

We created mutated plasmid with site directed mutagenesis kit as explained before.

Primer C338Y Forward	gaagtcctctcagagcgctattgggggacgatgc
Primer C338Y Reverse	gcatcgtcccccaatagcgctctgagaggacttc

Primer T341M Forward	cactgcctgcatcatcccccagcagcg
primer T341M Reverse	gcatcgtcccccaatagcgctctgagaggacttc

Tab 11: EPOR primers designed for the Site Directed Mutagenesis kit

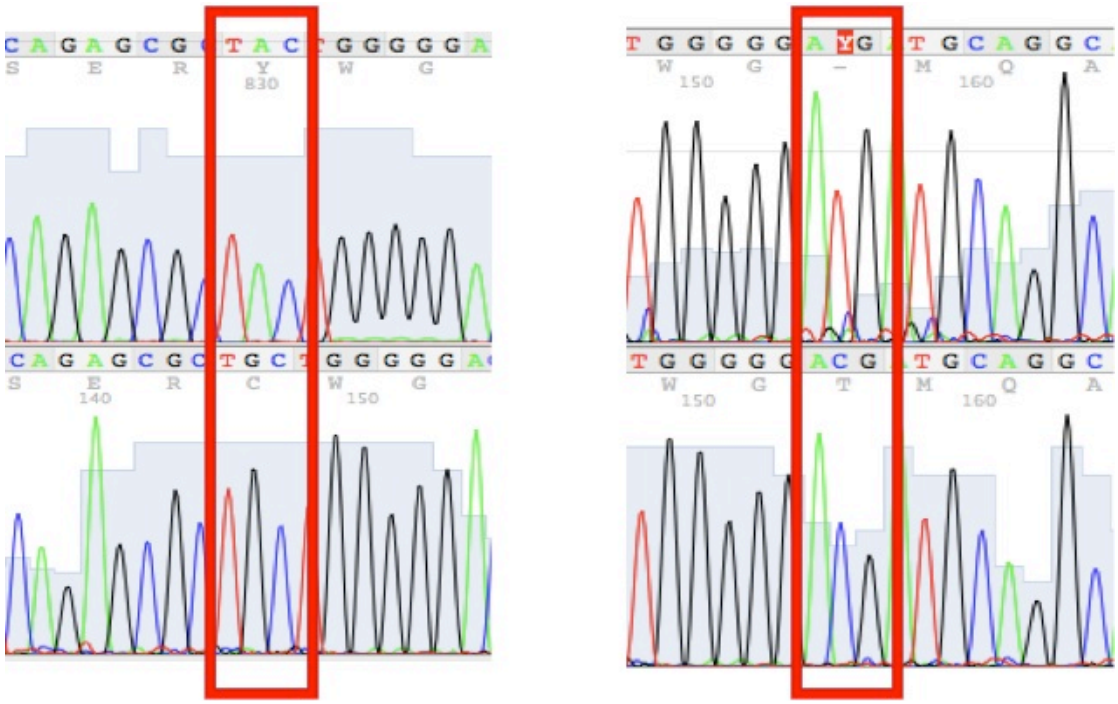

Fig. 16: Nucleotide sequences of the mutant EPOR plasmids.

After the site directed mutagenesis we executed a transient transfection of HEK293T cells: 24 hours after seeding 25 cm^2 plates, around 80% confluence, we performed the transient transfection using jetPEI (PolyPlus) according to the protocol given in table 12. JetPEI is very convenient because it allows to use the culture medium where cells grew even in presence of antibiotics.

1	Sol A:	250uL NaCl 150nM + 40 μL Jet PEI (40 μG/reaction)
2	Sol B:	250uL NaCl 150nM + 4 μG DNA
3		Mix Sol A + Sol B
4		Vortex 30"
5		incubation 10' at Room Temperature
6		add 500 μL of mix A+B per plate

Tab. 12: Transfection protocol with jetPEI chemical agent.

The day after we verified, with Fluorescence Activated Cell Sorting (FACS) technique, if the receptor was correctly expressed on the cell membrane (75). The FACS technique is a particular type of flow cytometry which allows, after staining with fluorescent antibodies, the detection of any epitope. The cells after being labeled pass through a capillary of the flow cytometer where they are excited by a laser which allows to detect whether the antibody has bound the epitope on the plasma membrane, and thus, in our case, if EPOR is expressed or not.

The experiment is then made by detaching the cells from the plate with PBS-EDTA, to avoid to ruin the membrane receptors, and putting $2x10^5$ cells for every transfected condition in a well of a 96 well plate. In this way we marked the four conditions (empty, wild type, MUT1 and MUT2) with a antibody for the control isotype mouse IgG2b Isotype Control (R&D Systems) and, in every different well, with an antibody specific to EPOR Human Erythropoietin Mab (R&D Systems). After a series of washes with cold PBS 1X the cells were resuspended and transferred into special tubes for the reading with Calibur flow cytometer (Becton Dickinson Bioscience).

With FACS we could notice that EPOR is expressed in about 50% of the transfected cells (Figure 17).

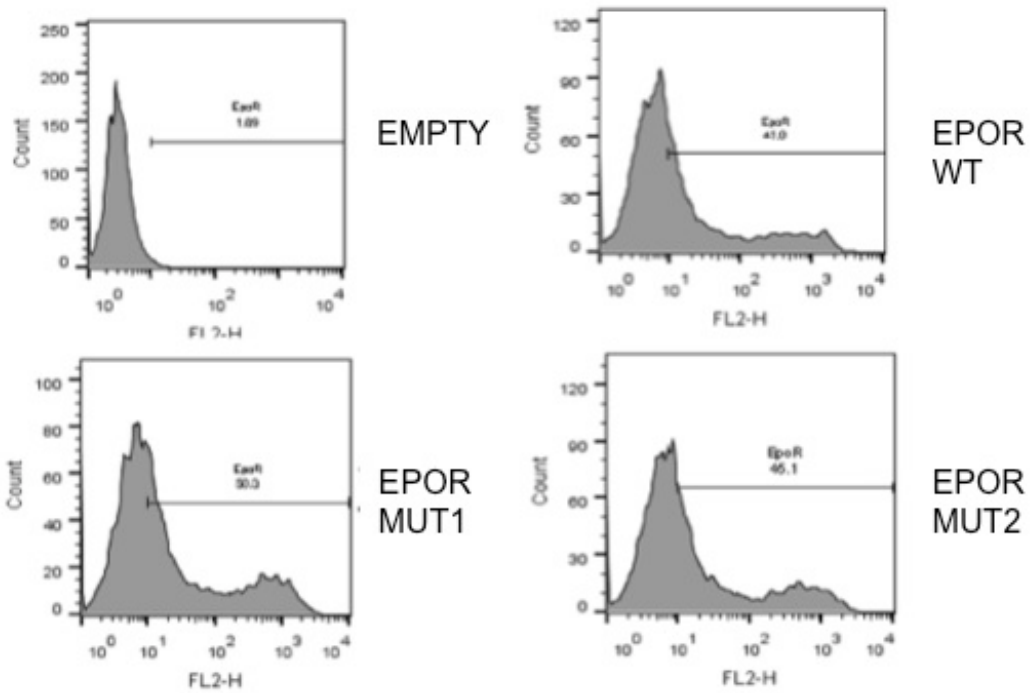

Fig. 17: FACS analysis Hek293T cells transfected with EPOR wild type e mutated

With the evidence of the correct expression of EPOR in transfected cells, a second transfection was performed according to the same protocol already described.

48 hours after the transfection we performed a kinetic of stimulation of the transfected cells in the different conditions (empty, WT, MUT1 and MUT2) with 3 U/ml of hrEPO (Novartis). The stimulation was stopped at time 0, 15, 30 and 45 minutes.

Below in table 13, the protocol used:

1	Morning: Change medium with 5mL DMEM without FBS
2	Afternoon: deteach cells with PBS-EDTA and put in Falcon
3	Centrifuge 5' at 1000 RPM
4	Wash with 4mL PBS 1X and centrifuge 5' at 1000 RPM
5	Resuspend in 4 mL of DMEM w/o FBS
	TIME COURSE
6	Add EPO (3U/mL) in falcon e mix by inversion
7	Stop the stimulation with 500uL cold PBS 1X at T0, T15, T30, T45.
8	Centrifuge at 4°C for 5' at 1000 RPM
9	Wash with 4mL PBS 1X and centrifuge 5' at 1000 RPM
10	Dry pellet: resuspend in 60uL of RIPA buffer (with NaF e Na3VO4) and transfer in 1,5 mL eppendorf
11	Vortex 2-3 min. and tranfer in ICE for 20'
12	Centrifuge at 4°C for 20' at 12000 RPM
13	Transfer the supernatant in 1,5 mL eppendorf
14	PROTEIN TITRATION

Tab. 13: EPO stimulation protocol of Hek293T cells.

Finally, after assessing the concentration of protein extracts with Pierce BCA Protein Assay Kit (Thermofisher), we developed Western Blot (WB) investigations analysis in order to detect if there are different activation states of the EPOR signaling pathway in all different points of the time course in presence or absence of stimulation:
The total protein presence (both activated or not) has been detected with αJAK2 antibody (Upstate), αSTAT5, αERK, αAKT (Cell Signalling) signal, using β-actin as control. We detected the activated proteins using primary antibodies α-phosphoJAK2, α-phosphoSTAT5, α-phosphoERK and α-phosphoAKT (Cell Signaling).
Unfortunately the WB data obtained, with this study approach, were almost null and confused although we changed several experiment variables (time of kinetic and EPO concentrations). Even by increasing the concentration of primary antibody and diluting it no longer in TBST 5% milk but in TBST 5% BSA or in TBST, in order to amplify the signal as much as possible, the results were always the same.
It was necessary to change strategy and to use a cell line that could be a study model with characteristics much closer to those *in vivo*.

K562 cells are an erythroid leukemia cell line with similar phenotype to the cells affected by the disease *in vivo* (76). This study model is much more truthful than the last one with HEK293 cells.
We grew K562 cell line in RPMI 1640 medium (EuroClone) supplemented with 10% FBS (EuroClone), 1% Pen Strep (EuroClone) and 1% L-Glutamine (EuroClone).
For the experiment we repeated all the steps of those studied for the HEK293T cells and also in this case the results were so confused for several reasons: for instance transfection with chemical reagents as jetPEI (PolyPlus) had a very low yield. However, we completed the experiment of kinetic stimulation but once again the results were not significant because very few cells expressed EPOR transfected.

For the following experiments we adopted electroporation to transfect K562 cells (77). Electroporation consists in a sudden electrical discharge operated in a cuvette which contains cells and DNA molecules in a liquid suspension. The electrical field opens simultaneously different transient pores in the cell membrane at numerous points, allowing the DNA molecules to penetrate (Figure 18). It is possible to optimize the electroporation parameters for the different types of cell lines by varying

the electric field intensity and the duration of the treatment. The area of the permeabilized membrane is directly proportional to the electric pulse intensity, while longer it lasts in time greater is the disruption of the area.

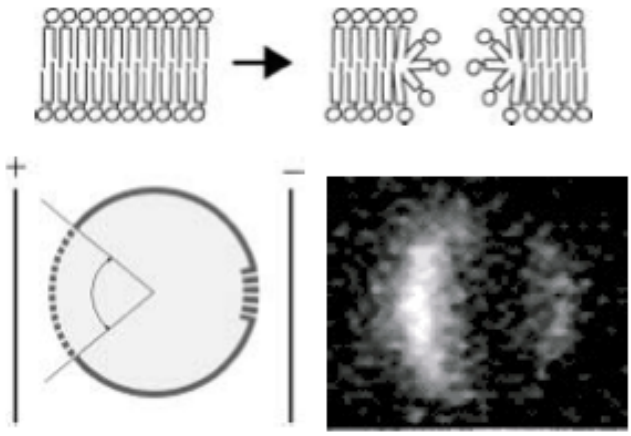

Fig. 18: transient pores formation on the cellular membrane after electroporation.

We tested the transfection efficiency with a plasmid encoding Green Fluorescent Protein (GFP), using different amounts of cells in the cuvette (1, 3 and 6 million cells). Electroporation was performed with an electroporator Nucleofector™ (Lonza) according to the protocol given by the company. In each cuvette we transfected the defined quantity of cells in 100 µL of Buffer A.

After 24 and 48 hours, we evaluated the amount of GFP expressed with a fluorescence microscope (Leica). We evidenced that the optimal transfection yield is with 2 µg of DNA per million cells in a total of 6 million cells (Figure 19).

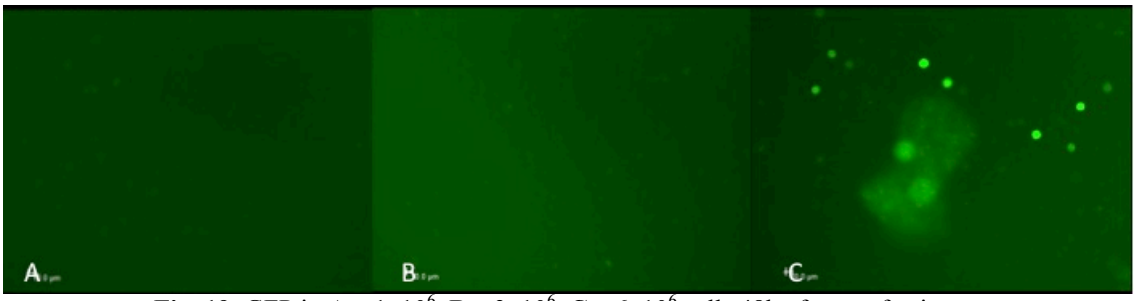

Fig. 19: GFP in **A** – $1x10^6$, **B** – $3x10^6$, **C** – $6x10^6$ cells 48h after trasfection.

Determined the amount of cells to transfect we proceeded with the electroporation of plasmids encoding EPOR WT and mutated. Once transfected we seeded 3 million cells, for every condition, in 25 cm^2 flasks in 5 ml of RPMI 1640 1% FBS.

We then analyzed the transfection yield of EPOR plasmids:
48h after the electroporation we extracted the RNA according to the protocol in table 14. with High pure RNA isolation kit (Roche).

1	Resuspend cells in 200 µl of PBS
2	Add 400 µl di Lysis/Binding Buffer and vortex for 15”
3	Transfer the sample in a High Pure Filter Tube
4	Centrifuge 15” at 8000 x g
5	Discard the flowthrough
6	Add a sol. of 90 µl Dnase I Incubation Buffer + 10 µl Dnase I and incubate 15’ at room temperature.
7	Add 500 µl Wash Buffer I and centrifuge 15” at 8000 x g. Discard the flowthrough (Repeat twice)
9	Add 500 µl Wash Buffer I and centrifuge 15” at 13000 x g. Discard the flowthrough
10	Elute with 50-100 µl of Elution Buffer e centrifuge 1’ at 8000 x g.
11	Store at -80° C

Tab. 14: RNA extraction protocol with High pure RNA isolation kit (Roche).

The RNA was then quantified with NanoDrop and later we performed a retrotranscription with a RT-PCR reaction. The mix and the thermal profile of the reaction are shown in table 15 and table 16 respectively.

iScript RT Supermix BioRad	4 µl
RNA	1 µg
Nuclease-free water	variable
Totale Vol.	20 µl

Tab. 15: RT-PCR reaction mix

	Temperature °C	Time min.
Priming	25	5
Reverse Transcription	46	20
RT inactivation	95	1

Tab. 16: RT-PCR thermal profile

At this point, obtained the cDNA, we executed a qPCR analysis. A relative quantification of mRNA of transfected EPOR had been carried out comparing with non-transfected cells. We used Actin as house keeping gene. The qPCR was performed with StepONE device (BioRad). Reaction mix and the thermal profile are shown in table 17 and 18 respectively.

SYBR Green Super Mix BioRad	10 µl
Primer Forward	2 µl
Primer Reverse	2 µl
cDNA	2 µl
H_2O	4 µl
Total Vol.	20 µl

Tab. 17: qPCR reaction mix.

T (°C)	**Time**	**Cycles**
95	3’	1
95	10-15”	35-40
T. annnealing and Extention	30-60”	
Melt Curve		
55 – 95 °C		

Tab. 18: qPCR thermal profile.

We made a relative quantification relating EPO WT and mutated threshold cycles, to those of Actin. After 48h the mRNA of different transfected EPOR conditions is expressed about 1800 times more than the sample not transfected. Figure 20 shows how the logarithmic phase of transfected samples amplification occurs at much lower cycles when compared to the sample not transfected.

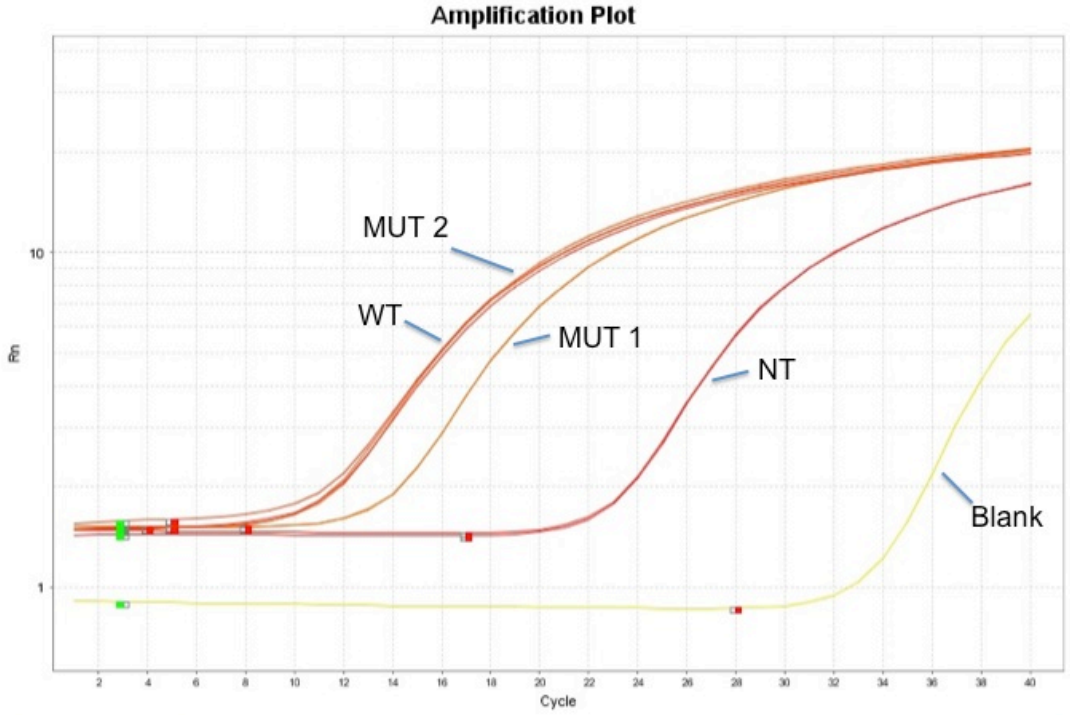

Fig. 20: Amplification curves of the qPCR reaction. Samples transfected WT and mutated (MUT1 and MUT2) display a logarithmic phase of *EPOR* amplification at much lower threshold cycles when compared to amplification cycles of non-transfected (NT) cells.

The week before the experiment we cultured cells in RPMI 1640 medium with only 1% FBS in order to synchronize them to be stimulated with EPO (Binocrit - Novartis) limiting as much as possible any disturbance derived from FBS.

Cells were then transfected with electroporation technique as already described, and 48h after transfection, we performed the stimulation kinetic according to the protocol in table 19.

STIMULATION KINETIC	
1	Transfer the cells Falcon
2	Add EPO (3U/mL) in Falcon and mix by inversion
3	Stop the stimulation with 500μL of cold PBS 1X at **T0**, **T5**, **T15**, **T30**, **T45**.
4	Centrifuge 5' at 1000 RPM
5	Wash with 4mL PBS 1X and centrifuge 5' at 4°C at 1000 RPM
6	Dry pellet: resuspend in 60μL RIPA buffer (with NaF e Na3VO4) and transfer in 1,5 mL Eppendorf
7	Vortex 2-3 min. and transfer on ICE for 20'
8	Centrifuge at 4°C for 20' at 12000 RPM
9	Transfer surnatant in a 1,5 mL Eppendorf
10	PROTEIN TITRATION

Tab. 19: EPO stimulation kinetic of K562 cells.

After the quantification of the protein extracts we performed WB analysis of various components of the EPOR signaling pathway such as JAK2, STAT5, ERK and AKT using the β-actin as house keeping. The SDS-PAGE was carried out in MES running buffer (Invitrogen) loading 35 micrograms of protein in NuPAGE Bis-Tris Mini gel (Novex).

Primary antibodies are αP-STAT5 a/b (Y694/Y699 R&D Systems), αSTAT5 (C-17 Santa Cruz), αP-Akt (S473 R&D Systems), αAkt 1/2/3 (H-136 Santa Cruz) , αP-ERK (E-4 Santa Cruz), αERK1 (K-23 Santa Cruz), αP-Jak2 (Y1007-1008 C80C3 Cell Signaling), αJak2 (D2E12 XP Cell Signaling), while for secondary antibodies we used IRDye 680RD Donkey αMouse (LI-COR) and IRDye 800CW Donkey αRabbit (LI-COR).

After incubation with secondary antibodies we developed the blot with Odyssey method (Li-Cor) which involves the use of a scanner Odyssey Infrared Imaging System (Li-Cor) whose secondary antibodies are IR sensitive.

We also analyzed the WB results with the software Image Studio developed by Li-Cor, to obtain the quantification of the signal. Furthermore it permitted us to implement the background reduction and adjust the brightness/contrast of the WB images.

RESULTS

Within the 106 patients (89 males, 17 females) *JAK2*-WT (Table 20) evaluated, in 97 we did not recognize any mutations in the indices genes nor we can identify any other case of erythrocytosis in the patient kindred: these patients were considered as sporadic IE.

	Not mutated	mutated
N° of patients (106 total)	97	9
Male (%)	81 (83,5)	8 (88,9)
Mean age (y)	52,8±16,4	24±20
Mean HB g/L	179,8	185,6
Mean HCT (%)	53,16	56,4
Mean WBC ($x10^9$/L)	7,5±2,7	8,1±3,7
Mean plts ($x10^9$/L)	222±67	236±74
Median EPOs (UI/L) (range)	11 (4-38)	52 (5-1280)
Patients with EPOs lower than normal limits (%)	24/73 (32,8%)	3 (33%)
Patients with EPO higher than normal (%)	49/73 (66.2%)	6 (66%)

Tab. 20: Main clinical and laboratory data at diagnosis of our patients.

Nine patients had one of the mutations of *EPOR* or of OSP genes: 2 in *EPOR* gene, 2 in the *VHL* (2 other patients had classical VHL R200W), 2 in *HIF2α*, and a 1 in PHD2 (Table 21).

	N° of patients	New mutations	Yet known mutations
VHL	4	2 - (R82C - G144R)	2 - (R200W)
PHD2	1	1 - (G349S)	/
HIF2α	2	2 - (R550W - R602Q)	/
EPOR	2	2 - (C338Y - T341M)	/

Tab. 21: Mutations found within 106 patents with not-MPN erythrocytosis

The two missense mutations in the *VHL* gene map in respectively exon 1 and exon 2, in a region that encodes the interaction domain with HIF2α. The variation c.244C>T - p.Arg82Cys mutation induce a change between a positively charged amino acid and a polar one. The mutation c.430G>A - p.Gly144Arg causes the substitution of a positively charged amino acid with a polar amino acid (78) (Figure 21).

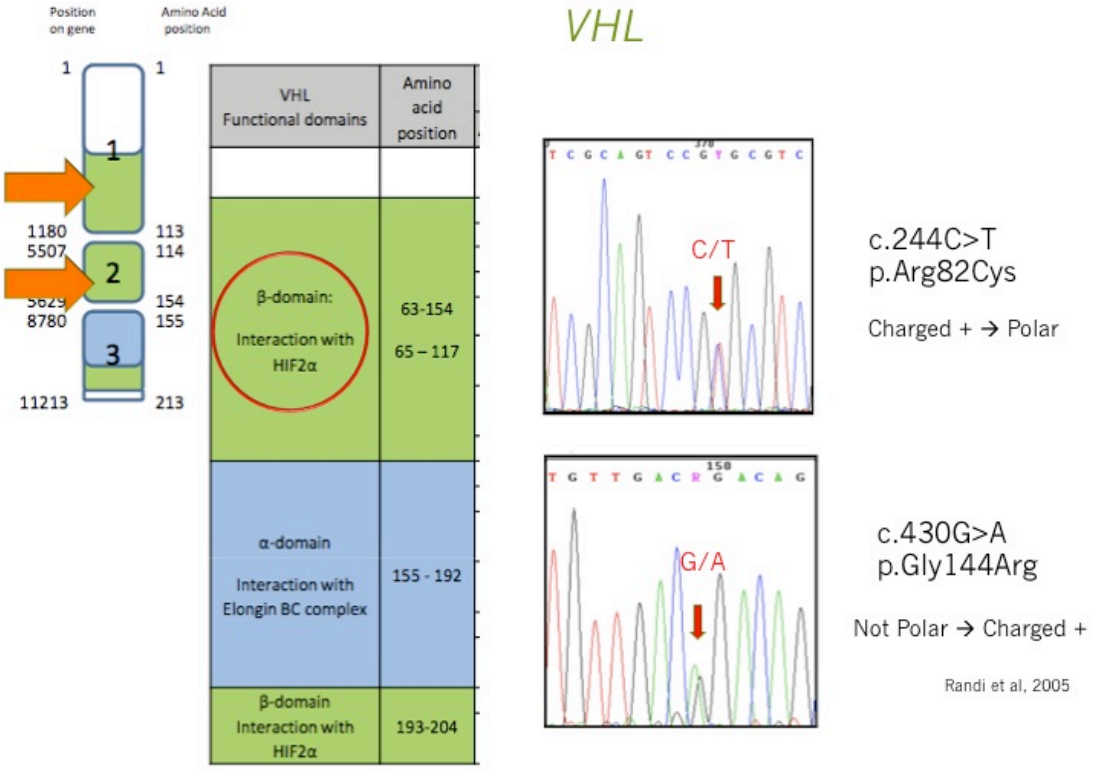

Fig. 21: Mutations detected in functional domain of *VHL*.

We found two missense mutations in *HIF2α* gene (c.1648C>T – p.Arg550Trp and c.1805G>A – p.Arg602Gln) both mapping in exon 12. This exon encodes the domain hydroxylated by PHD2. We have not at present evaluated the effect of these mutations on the protein function.

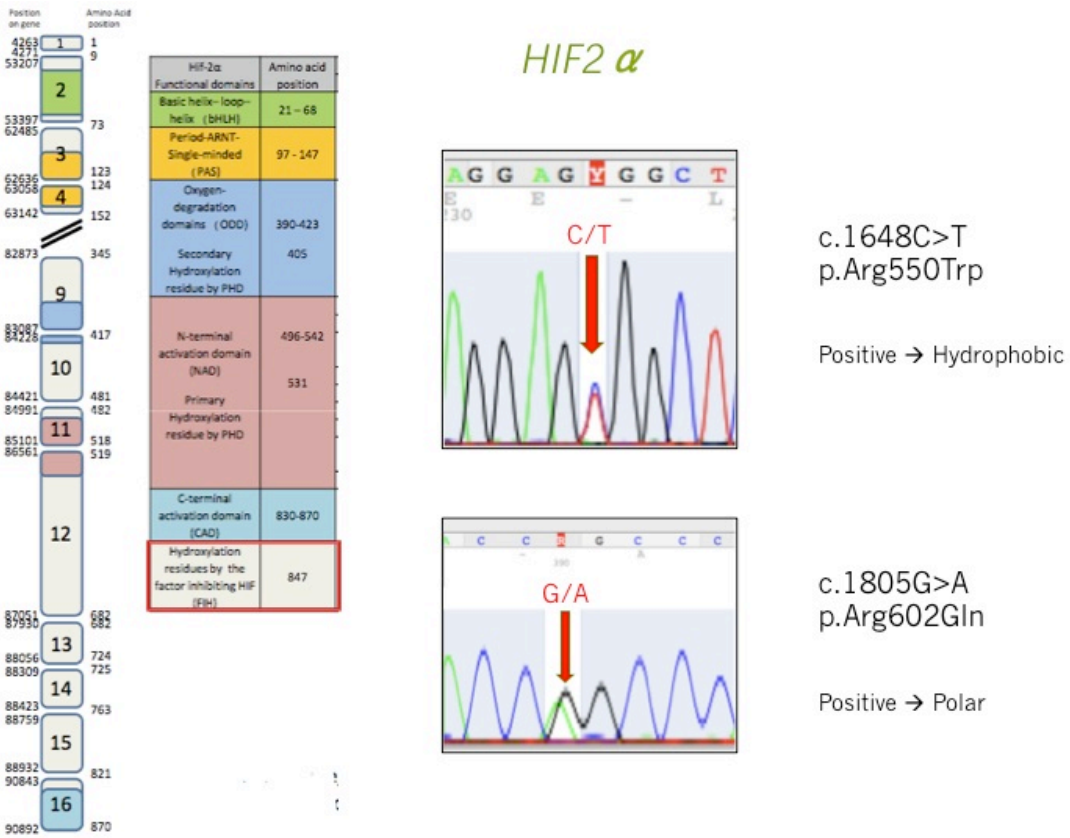

Fig. 22: 2 missense mutations detected in exon 12 of *HIF2α* gene.

The mutation c.1045G>A – p.Gly349Ser in *PHD2* gene is located in exon 3 that encodes part of the dioxygen domain responsible for the binding of with O_2 molecule. This binding allows the hydroxylation of HIF2α and therefore its degradation (Figure 23).

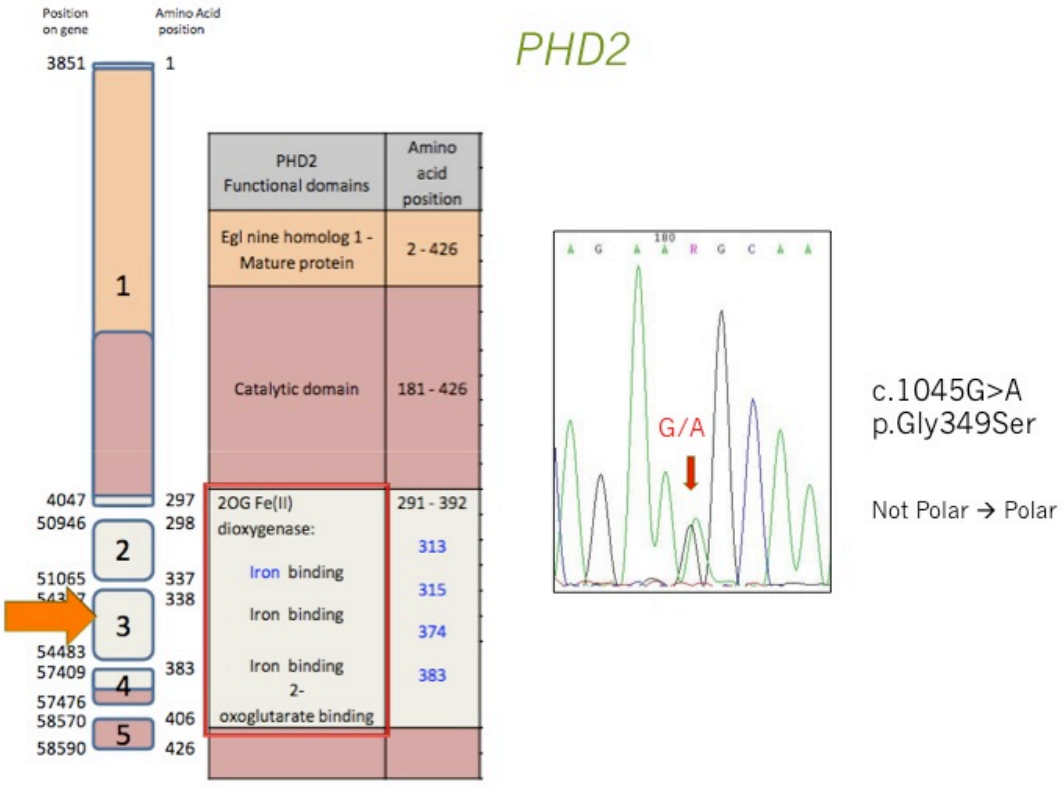

Fig. 23: *PHD2* gene and the missense mutation found in exon 3.

In *EPOR* gene, we detected 2 missense mutations both in exon 8 that encodes for a negative regulatory domain. The first mutation (c.1013G> A – p.Cys338Tyr) changes from a polar to an aromatic amino acid, and the second one (c.1022C>T – p.Thr341Met) leads to a non-polar instead a polar amino acid (Figure 24).

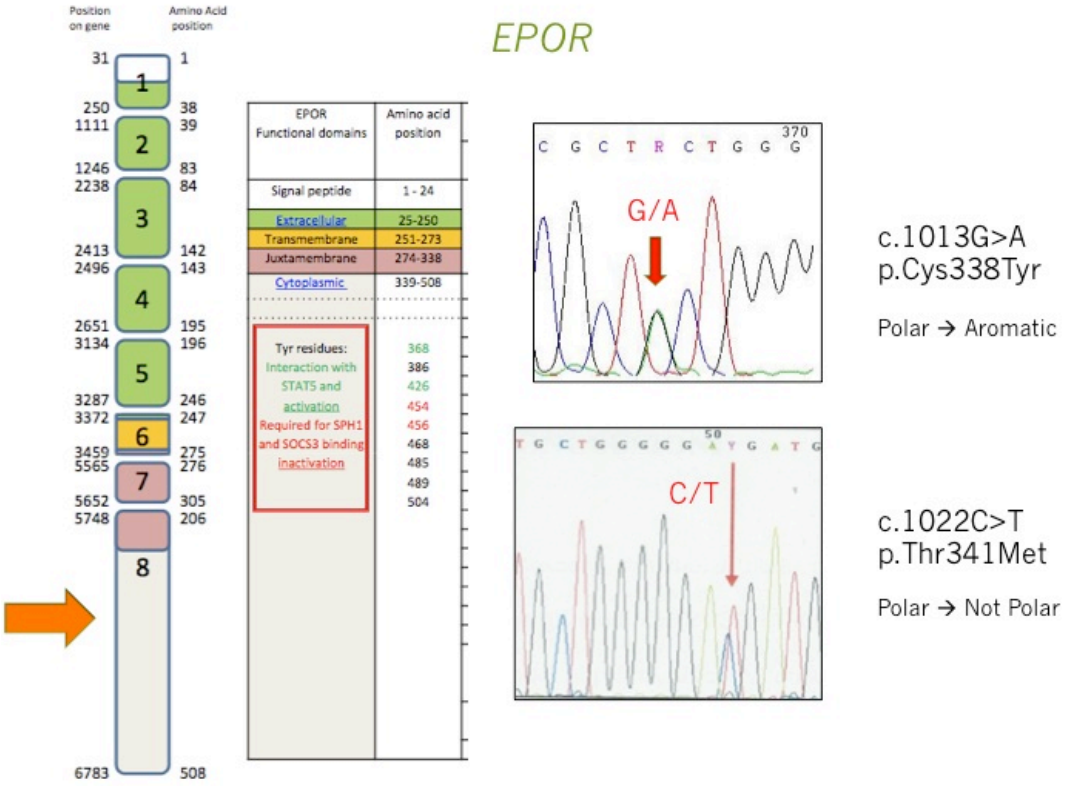

Fig. 24…: Functional domains of *EPOR* and the mutations found during the genetic study.

FUNCTIONAL STUDY OF THE NOVEL *PHD2* MUTATION

The data emerged from the *in silico* study showed that the nucleotide variation c.1045G>A in *PHD2* is not particularly compromising for the protein if compared to other more severe mutations (Figure 25).

Fig. 26: Comparison with the new G349S mutation, found in our patients, with Q157H, P200Q and R371H non-severe mutations and D254H e H374R defined as severe.

However, considering that the mutant residue maps in a highly conserved domain present in many animal kingdoms, we decided proceed to the functional studies anyway (Figure 26).

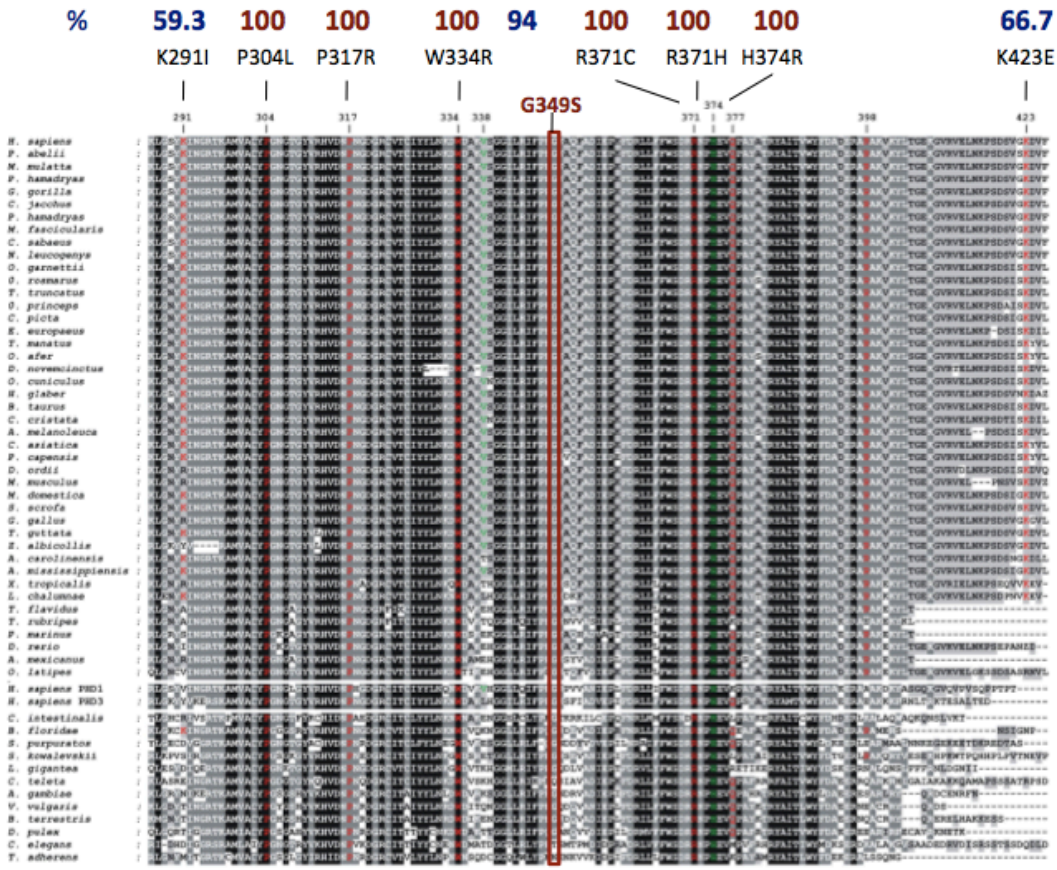

Fig. 26: The alignment of the various isoforms of PHD2 in the various animal kingdoms shows that the mutation G349S maps in a widely conserved domain.

The data obtained from the DLR *in cellulo* assays show that the mutation PHD2 G349S does not display relevant differences compared to the wild type protein.

In fact, as can be seen in figure 27, the luciferase activity in cells transfected with PHD2 G349S is comparable to that of PHD2 WT cells: the mutated PHD2 enzyme did not developed a big luciferase activity due to lack of hydroxylation of HIF, as seen with the control (a patient carrying D254H mutation).

We found the same results for the experiments conducted in cell line REPC-Sh-PHD2 (Figure 28).

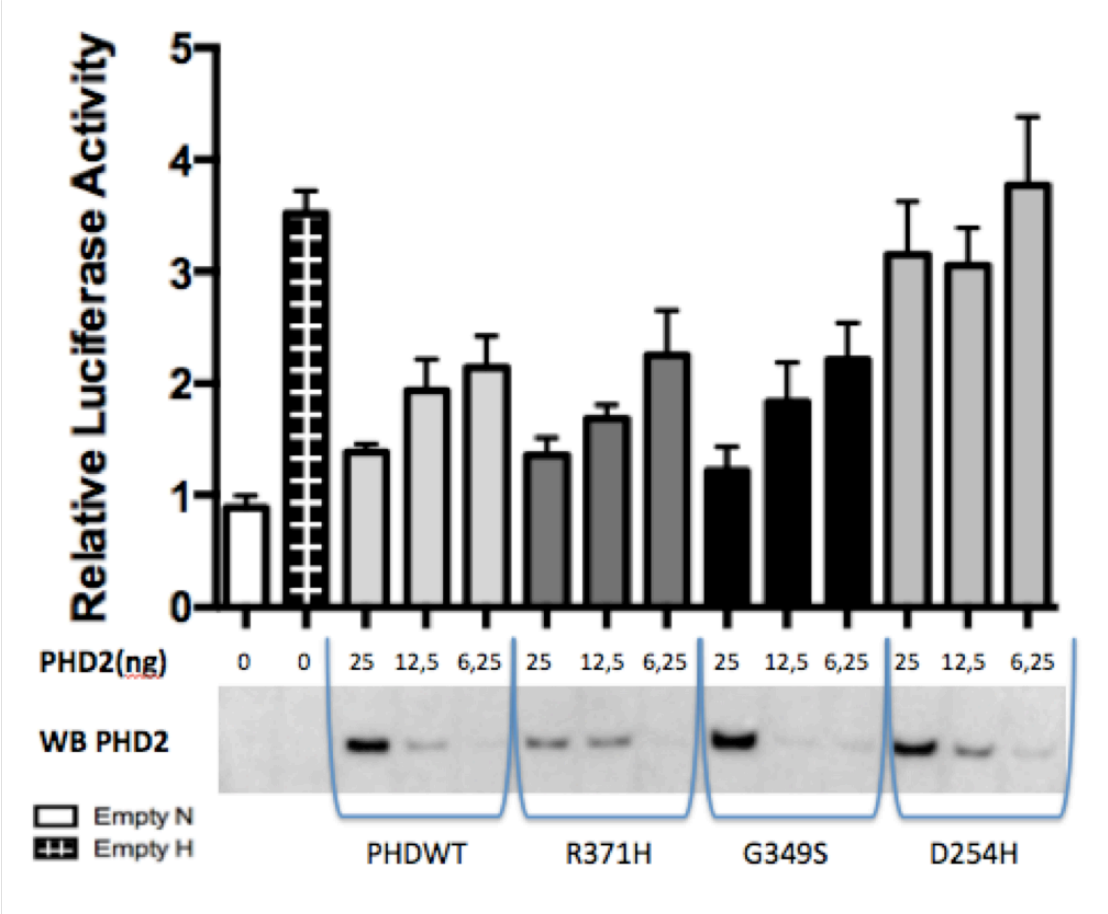

Fig. 27: Dual Luciferase Reporter Assay in Hek293T cells transfected with several amount of mutant (R371H as severe and D254H as not-severe) or *PHD2 WT* vector. Under the graph there is a blot showing the amount of enzyme PHD2 produced by the transfected cells.

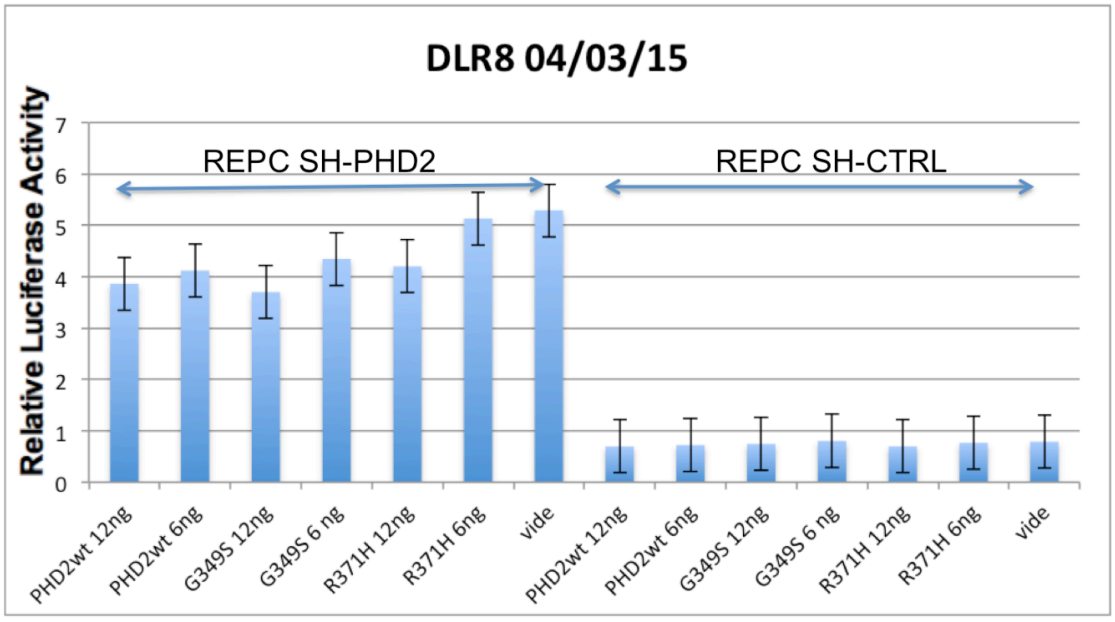

Fig. 28: Dual Luciferase Reporter Assay in REPC cell line silenced for *PHD2* and transfcted with several amount of mutant or *PHD2* WT vectors.

The *in vitro* hydroxylation test did not show differences of PHD2 enzymatic activity between the mutated protein and the wild type one: the WB analysis reveal that the amount of hydroxylated HIF-ODD is almost similar with PHD2 WT and with PHD2 G349S. In contrast, with the negative control (PHD2 P317R) hydroxylated HIF-ODD is totally absent (Figure 29).

Fig. 29: Western Blot analysis of the hydroxylation test in which an antibody specific for HIF-ODD hydroxylated had been used.

FUNCTIONAL STUDY OF THE *EPOR* MUTATIONS

The study *in silico* indicates that EPOR c.1013G>A and c.1022C>T mutations are not severe: however, the missense mutations with aminoacid changes can compromise the protein characteristics: Especially c.1013G>A mutation may affect the splicing mechanism (Figure 30) and then the correct maturation of mRNA.

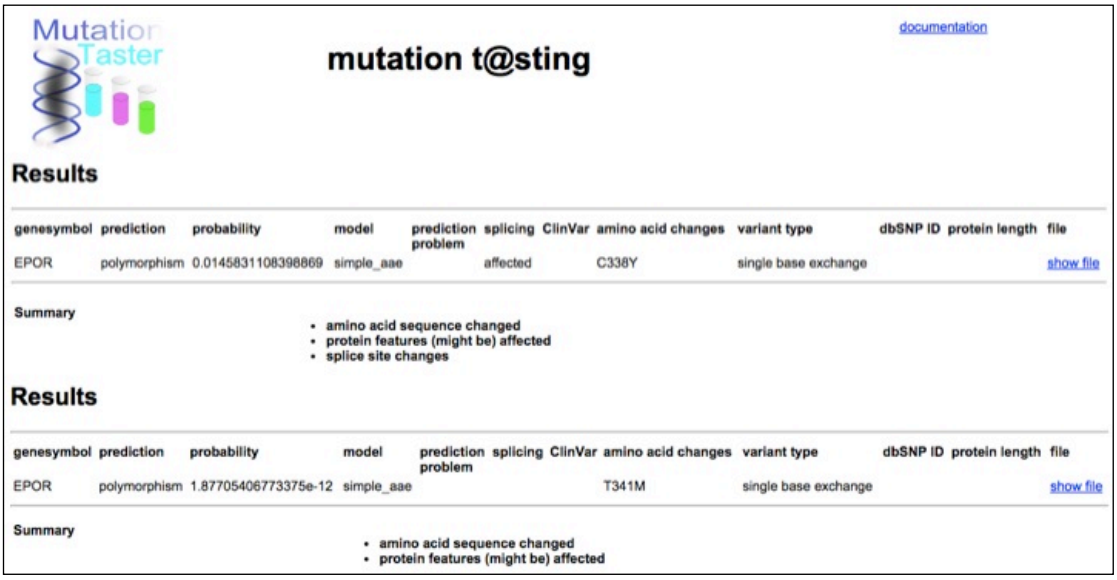

Fig. 30: Computational study conducted for the *EPOR* gene mutations. In both mutations there is an amino acid change with possible impairments of the characteristics of the protein. For the mutation c.1013G>A is also provided a variation of the splicing site.

WB analysis (Figure 31) display that the EPOR signaling cascade is more active in mutated cells than the WT when stimulated with EPO. The signal transducer STAT5 is more active in C338Y cells than the T341M at 5 to 15 minutes after the stimulation with the cytokine.

The mitogen-activated protein kinase ERK appeared more active in the mutated conditions compared to the WT at the point T0 and the same activating condition is in T5, and also in the other points of the time course suggesting that the receptor is constitutively active in absence of EPO stimulation. Mild activation in mutant conditions, had also been detected in T15 and T30. These results are also confirmed with the WB signal quantification made with the sofware Image Studio powered by Li-Cor (Figure 32).

The phosphorylated transcription factor Akt is very weak just in step T5 (doubtful interpretation). No activation signal was detected in kinase JAK2.

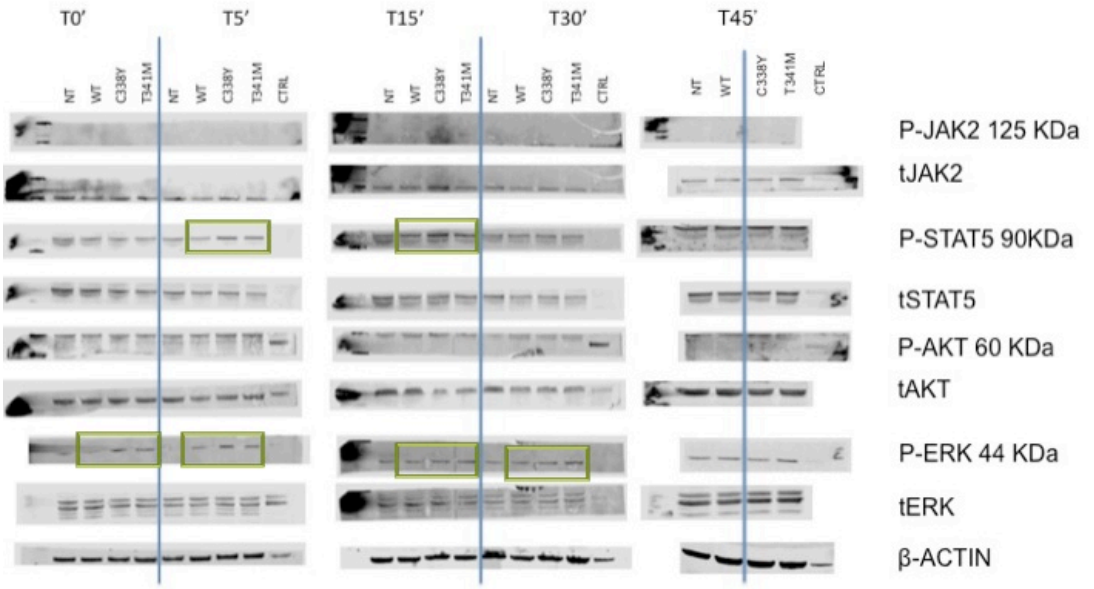

Fig. 31: The EPOR signaling cascade resulted more active in mutated cells than in the WT when stimulated with EPO. 5' and 15' after stimulation, transfected cells with C338Y mutation showed higher phosphorylation of STAT5 compared to T341M mutation.
We observed also a similar phosphorylation pattern of ERK occurring earlier than STAT5 (at 0' and 5'), while no activity signals were detected in JAK2 and AKT cascades.

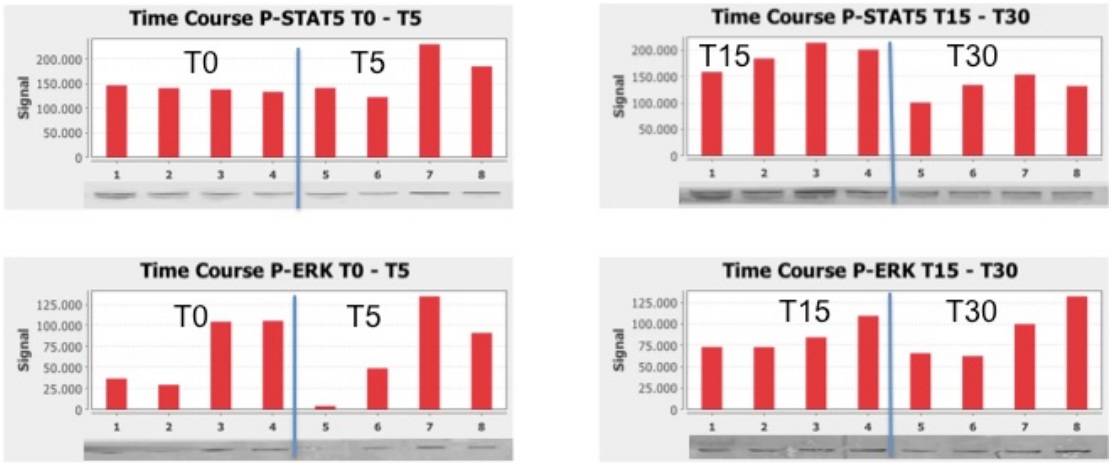

Fig. 32: Quantifications of WB signal confirm the major activation of the signal pathway in mutated condition (3,4 and 7,8) compared to the not transfected (1 and 5) and the wild type (2 and 6). Furthermore P-ERK quantification is more relevant in mutated conditions already in absence of EPO suggesting the constitutively activation of EPOR.

DISCUSSION

Erythrocytosis is a relatively rare finding in the clinical practice. Most studies on this topic are devoted to the patients with PV. These patients are affected by a neoplasms, their history is characterized by frequent thrombotic events and they may evolve in myelofibrosis and acute leukemia. Therefore, most Hematologists have been attracted by this disease (18).

In contrast, CE are extremely rare diseases (56). They can be either primary or secondary disorder, depending by the gene involved: the mutations in OSP genes are considered secondary disorders, because they induce an increased EPO production that stimulates the erythroid proliferation (29). In contrast, mutations in *EPOR* gene directly interest the erythroid progenitors and therefore are classified as primitive disorders (28).

In our Center, we meet many patients with erythrocytosis. When we exclude in sporadic cases a PV not finding JAK2 V617F or *JAK2* ex 12 mutations, we perform the germinal DNA investigation on the basis of EPOs level: in the presence of high EPOs, we explore the genes of the OSP and if EPOs is low we study the *EPOR* gene.

We found that it is possible to recognize rare mutations not only in familial cases but also in sporadic patients. In the past, we published the data of a child with very high HCT carrying a never previously published *VHL* mutation (78). The mother and one of the sisters had the same mutation but they do not have the phenotype

All the mutations we found are heterozygous and our previous experience in a *VHL* mutated patient induced us to evaluate *EPOR* and OSP genes in a large cohort of otherwise considered IE.

Within 106 cases, we found 9 gene mutations [4 mutations of *VHL* (2 classical Chuvash mutation, 1 yet published by us and 1 never described), 1 new mutation in *PHD2*, 2 mutations in *HIF* never described and 2 novel mutations of *EPOR*] on a whole accounting for 8,5% of all IE followed in our centre.

The present study deal with one patient carrying a new *PHD2* mutation and two unrelated patients with 2 different *EPOR* mutations. None of these 3 patients have available relatives to study, and only in one with *EPOR* mutated gene we were informed that his died father had “thick blood”. Therefore, we had no chance to

evaluate the kindred of these patients and they result “sporadic” at least from a clinical point of view.

Here, we report functional evaluation of the proteins produced by mutated *PHD2* and *EPOR* gene. These three mutations have never been previously described.
The *PHD2* mutation we found is a missense mutation (c.1045G>A - p.Gly349Ser) (79, 80). It does not induce a clear loss-of-function, while PHD2 D254H and H374R mutations showed a functional activity of the enzyme. Other studies described that mutations Q157H, P200Q and R371H are not active (42, 81). Therefore, these mutations as well as our one are weaker than the first described and more sensitive methods should be used to evaluated their function.
We can also suppose that, in the case of weak mutations, other genes may be involved in the development of erythrocytosis. Unfortunately, the absence of an evaluable kindred did not facilitate the identification of the targeted genes that should be investigated after surveys by Next Generation Sequencing (NGS).

In *EPOR* gene, we found two different mutations: c.1013G> A - p.Cys338Tyr and c. 1022C>T - p.Thr341Met (82). The two new mutations of the *EPOR* gene here reported represent about 8% of already published *EPOR* mutations. They are located in exon 8 as all the 26 previous described functional mutations and impair the C-terminal negative regulatory domain resulting in a gain-of-function in the EPOR signaling cascades both in C338Y and in T341M mutation.
These novel mutations, causing hyperactivity, increase proliferation and differentiation (effect of ERK pathway), and decrease of apoptosis of erythroid progenitor (effect of STAT5 pathway), sustain the erythrocytotic phenotype of our patients. The future evaluation of the effect on NFKB and on GATA1 should confirm the anti-apoptotic effect of the mutations.
We underline that these mutations are both missense and no previous descriptions are available in the literature about functional effect of missense mutations on the signal cascade of *EPOR* signaling.

This study give a contribute to the knowledge of CE. Significant advances have been made during the past decade in this filed (83) and the incoming use of NGS is

expected to further expand the number of genes involved in erythrocytosis, causing a reduction of idiopathic forms (84).

Our study also shows that erythrocytotic sporadic patients may have a mutation in the presently known genes involved in erythrocytosis, and we suggest to explore these genes in all patients with erythrocytosis when a clear ethiology is not recognized, even if this is an evident economical aspect that must be considered.

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