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# EXCESSIVE ERYTHROCYTE PPIX INFLUENCES THE HEMATOLOGIC STATUS AND IRON METABOLISM IN PATIENTS WITH DOMINANT ERYTHROPOIETIC PROTOPORPHYRIA

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**Abstract –** Partial deficiency of the last enzyme of the heme biosynthetic pathway (namely ferrochelatase, FECH) in humans is responsible for erythropoietic protoporphyria (EPP). This disorder is characterised by painful photosensitivity, due to excessive production of protoporphyrin IX (PPIX) by erythrocytes. Controversial hypotheses have been proposed to explain the hematologic and iron status of EPP patients. In the present work, we explored these parameters in 55 patients with dominant EPP recruited at the French Center of Porphyrias (Colombes, France) and confirmed by molecular analysis. Our data show that erythrocyte accumulation of PPIX in EPP patients influences hematologic and iron status. Patients studied had a mild anemia and thrombocytopenia, as shown by the downward shift of hematologic parameters, which positively correlated with the amount of erythrocyte PPIX. Interestingly, erythropoiesis did not seem to be limited by iron supply in patients, since serum iron and soluble transferring (Tf) receptor (sTfR) were normal. However, iron and Tf saturation negatively correlated with erythrocyte PPIX. Moreover, and as previously described in a mouse model of EPP, we noted a positive correlation between erythrocyte PPIX and Tf levels. Altogether, these results suggest a positive effect of PPIX on the synthesis on Tf, which could facilitate the mobilization of tissue iron stores to meet erythropoiesis requirement. Based on these observations and previous results in EPP mouse model, we propose that the PPIX–liver transferrin pathway plays a role in the orchestration of iron distribution between peripheral iron stores, the spleen and the bone marrow.

**Key words:** Liver, Protoporphyrin IX, Ferrochelatase, Erythropoïetic Protoporphyria

## INTRODUCTION

Erythropoietic protoporphyria (EPP, Mendelian Inheritance in Man [MIM] 177000) is an inherited disorder of heme biosynthesis that results from a partial deficiency of ferrochelatase (FECH, EC 4.99.1.1.), the final enzyme of heme biosynthetic pathway [22; 24]. FECH is an inner membrane mitochondrial enzyme which catalyses the insertion of ferrous iron into protoporphyrin IX (PPIX) to form heme. Its deficiency in bone marrow erythroid cells leads to increased synthesis of PPIX that accumulates in the bone marrow, erythrocytes, liver, plasma and skin [6]. Usually, EPP is inherited in an autosomal pseudo dominant manner, in which a common intronic single-nucleotide polymorphism (SNP), IVS3-48C, is inherited *in trans* to a dominant mutation [12] that abolishes or markedly decreases FECH activity [13].

The most common clinical manifestation of EPP is lifelong acute, painful photosensitivity of sun-exposed skin due to the photodynamic action of PPIX [5]. In addition, accumulation of PPIX in the liver may result in hepatic complications, such as cholelithiasis or fatal liver failure (in about 2 % of patients) [20; 25-26].

Mild anemia is observed in 20 to 50 % of EPP patients [7; 23], but the pathogenesis of the hematologic symptoms is not yet fully understood. The pathophysiological feature of the human disease can be studied using the homozygous mutant mouse *Fech*$^{m/Pas}$, which shows a residual FECH activity of 5 % in the liver and spleen, skin lesions and hepatic

dysfunction with PPIX deposits [1; 4; 28]. Interestingly, investigation of iron metabolism in these mice has lead to the observation that if they develop anemia, their total body iron is normal and is redistributed from peripheral tissues to the spleen [1; 19]. In addition, these mice showed an increase of plasma Tf [19], suggesting that erythrocyte PPIX could act as a sensor of iron to stimulate the synthesis of hepatic Tf in situation of anemia (probably through an indirect pathway), therefore increasing the iron supply for erythroid cells.

Although anemia in EPP patients probably reflects the lack of FECH activity to form heme, the precise mechanisms involved in such disorder and its relationship with iron metabolism remain elusive. In order to precise theses points, we investigated the hepatic, lipidic, hematologic and iron status of a well-defined group of 55 patients from EPP families recruited at the French Center of Porphyrias (Colombes, France).

## MATERIALS AND METHODS

### *Patients recruitment and molecular diagnosis*

All the procedures involving human subjects were performed in accordance with the 1983 revision of the Declaration of Helsinki, and the study was approved by the Hospital Ethics Committee of Hôpital Ambroise Paré, in Paris. Normal values for haematological, hepatic and lipid parameters have been established in a control group of healthy blood donors or children matched in sex and age.

The cohort consisted in 55 subjects (age ranging between 12 and 45 years old). from EPP families recruited at the French Center of Porphyrias (Colombes, France) between 1990 and 2007. All the subjects presented a documented clinical and laboratory history, and a high level of free protoporphyrin in their erythrocytes (52,292 ± 37,580 [mean ± SD] nM; normal <1,900 nM). All these patients had no associated etiology for iron deficiency and/or liver dysfunction and never received iron supplementation. Molecular analysis confirmed the diagnosis of the disease in these patients.

### *Lymphocyte FECH Enzyme Assay and Erythrocyte Protoporphyrin Measurements*

We assayed peripheral lymphocyte FECH enzyme activity in all the patients. FECH activity was determined by fluorometric measurement of zinc-mesoporphyrin formation after incubation for 60 min at 37°C, as previously established [18], with some modifications. For routine assays, a peripheral lymphocyte homogenate was prepared in 50 mM Tris-HCl (pH 7.6) and 20% glycerol, and the protein concentration measured using the Bradford method (Bio-Rad reagents). The reaction consisted of a 5-min preincubation period at 37°C, with 200 µl of lymphocyte homogenate, 200 µl of incubation buffer (250 mM Tris-HCl [pH 7.6], 1% [v/v] Triton X-100, and 1.75 mM palmitic acid), and 40 µl of 0.5-mM mesoporphyrin (final concentration 43 µM). Then, 20 µl of 1-mM zinc acetate solution was added (final concentration 43 µM), and the incubation continued for a further 60 min. A blank was prepared without the cell homogenate. The reaction was stopped by the addition of a dimethyl sulfoxide/methanol mixture (30:70 [v/v]). After centrifugation, the supernatant was transferred to a fluorometry cell, and the fluorescence was measured at 580 nm, with an excitation wavelength of 410 nm. The enzymatic activity was expressed as nanomoles of zinc-mesoporphyrin formed per hour per milligram of protein at 37°C (normal value = 4.83 ± 0.91 [mean ± SD] nmol mesozinc/h/mg). The erythrocyte protoporphyrin (PPIX) was determined by standard methods [24].

### *Hematological, iron status and biological parameters*

All analysis was carried out in the same laboratory. Hematocrit (Ht), hemoglobin (Hb), red blood cell count (RBC count), mean cell volume (MCV) and platelets were determined by standard automated methods (Siemens company). Transferrin (Tf), transferrin saturation (Tf saturation), iron binding capacity of transferrin (IBCT), ferritin and iron were also measured (Dade Behring, RxL max). sTfR were determined by immuno-nephelometry (Siemens medical healthcare, BN Prospec). Hepatic parameters (alkaline phosphatase, total bilirubin, Gamma GT, SGOT/ASAT and SGPT/ALAT) and lipid parameters (triglycerides, cholesterol, cholesterol HDL and LDL) were determined by standard automated methods (Dade Behring, RxL max).

### *Statistical methods*

Data were analysed using Microsoft Excel software. Results are expressed as mean ± standard deviation for normally distributed data. Correlation studies were performed by linear regression using Statview software. Statistical significance of correlations was evaluated using the non parametric Spearman test. Statistical results were corrected using the Bonferroni correction for multiple analyses.

## RESULTS

### *Patients and porphyrins analysis*

All the patients recruited at the French Center of Porphyrias (Colombes, France) presented a clinical and laboratory history of EPP, and their molecular diagnosis confirmed that 39 of them had one *FECH* mutation with one or two *FECH* IVS3-48C alleles. As shown in table 1, erythrocyte PPIX is increased in all subjects with low FECH activity (<3.5 nmoles of ZnPPIX/hour per milligram of protein). Consistent with previous report [14], PPIX concentration was higher in men than in women, although not being statistically significant.

### *Hematological analysis and iron metabolism*

Results of hematological analysis are shown in table 2. Ht, Hb concentration, RBC count and MCV in the EPP patients were towards the lower end of the reference values, particularly in women. This hypochromic microcytic anemia is mild. Only two women had an Hb below 10g/dl, and none of the men had an Hb below

**Table 1.** Ferrochelatase activity and erythrocytes PPIX in patients with EPP.

| | patients EPP | | | reference value |
|---|---|---|---|---|
| | all (n=55) | women (n=27) | men (n=28) | |
| **FECH activity** [a] | 1.86 ± 0.89 | 1.8 ± 0.59 | 1.68 ± 0.68 | > 3.5 |
| **Erythrocyte PPIX** [b] | 52292 ± 37580 | 48178 ± 27333 | 54557 ± 45177 | <1900 |

Ferrochelatase (FECH) activity and erythrocytes PPIX were evaluated in 55 patients with EPP.
Data are expressed as mean ± SD.
a: in nmoles of ZnPPIX/hour per milligram of protein measured in peripheral lymphocytes.
b: in nmoles/liter.

**Table 2.** Hematologic and iron parameters in patients with EPP.

| | patients EPP | | | reference value |
|---|---|---|---|---|
| **Hematologic parameters** | all (n=28) | women (n= 14) | men (n= 14) | |
| **Ht * (%)** | 36.4 ± 4.6 | 35.6 ± 4.6 | 37.3 ± 4.5 | 33.0 - 43.0 |
| **Hb level (g/dL)** | 12.3 ± 1.3 | 11.8 ± 1.2 | 12.7 ± 1.2 | 11.5 - 16.0 |
| **RBC count (x $10^{12}$/L)** | 4.5 ± 0.5 | 4.3 ± 0.4 | 4.7 ± 0.4 | 4.00 - 6.00 |
| **MCV (x $10^{15}$ L)** | 80.5 ± 11.6 | 78.1 ± 15.3 | 82.9 ± 5.6 | 77 - 100 |
| **Platelets (x $10^{9}$/L)** | 201.9 ± 68.3 | 192.6 ± 62.0 | 211.2 ± 75.3 | 150 - 400 |
| **Iron parameters** | all (n=55) | women (n= 27) | men (n= 28) | |
| **Tf (g/L)** | 2.82 ± 0.58 | 2.99 ± 0.62 | 2.65 ± 0.50 | 2.00 – 4.00 |
| **Tf saturation (%)** | 22.9 ± 12.1 | 21.7 ± 10.6 | 24.2 ± 13.2 | 20 - 45 |
| **sTfR ** (mg/L)** | 1.12 ± 0.13 | 1.09 ± 0.14 | 1.15 ± 0.10 | 0.8 - 1.6 |
| **IBCT (µmol/L)** | 70.5 ± 14.6 | 74.9 ± 15.6 | 66.3 ± 12.3 | 50 - 70 |
| **Ferritin (µg/L)** | 32.8 ± 37.3 | 22.9 ± 18.9 | 42.2 ± 47.4 | 8 – 100 |
| **Iron (µM//L)** | 15.5 ± 7.4 | 15.6 ± 6.9 | 15.5 ± 8.0 | 10 – 26 |

Hematologic status (hematocrit [Ht], hemoglobin [Hb], erythrocyte [RBC] count, mean corpuscular volume [MCV] and platelets)
was evaluated in 28 to 40 (*) patients with EPP. Iron status (transferrin [Tf], Tf saturation, serum Tf receptor [sTfR], Iron Binding Capacity of Tf [IBCT], ferritin and iron) was evaluated in 55 or 27 (**) patients with EPP.
Data are expressed as mean ± SD.

11g/dl. The defect in erythropoiesis seems to increase with the level of PPIX, although this was not statistically significative ($r^2$ = 0,145 p = 0,019 Figure 1A). As shown in table 2, the soluble transferrin receptor (sTfR) was 1,12 ± 0,13 mg/mL and iron is normal in patients EPP. These results suggest that the defect in erythropoiesis affecting hemoglobinisation in these patients is not the consequence of limited iron supply.

Interestingly, patients also present a mild thrombocytopenia (table 2), and correlation studies showed a highly significant and negative correlation between erythrocyte PPIX and platelets ($r^2$ = 0,395 p = 0,0002, Figure 1A).

As shown in table 2, Tf saturation and ferritin were towards lower values in patients. These both parameters positively correlated with Hb in patients (not shown). In addition, and as expected in iron-deficient anemia situation [8], linear regression shows that iron and Tf saturation inversely correlates with erythrocyte PPIX of patients ($r^2$= 0,219 p=0,0005 and $r^2$= 0,309 p<0,0001, respectively, Figures 1B). As shown in figure 1B, we also noted a significative increase of Tf with the amount of erythrocyte PPIX in EPP patients ($r^2$=0,194, p = 0,0006). On the other side, there was no significative correlation between ferritin and PPIX. Altogether, these results suggest mild iron depletion in patients with EPP.

### *Hepatic and lipidic metabolism*

Because PPIX accumulates in hepatocytes, chronic liver disease may be observed in EPP patients [21]. Therefore, we evaluated hepatic parameters in our cohort, such as alkaline phosphatase, total bilirubin, gamma GT, ASAT and ALAT (table 3). One or more of these parameters was increased in 39 (71%) patients (12 for alkaline phosphatase, 4 for total bilirubin, 14 for gamma GT, 9 for ASAT, 36 for ALAT). Anyway, no significant correlation was observed between any of these parameters and erythrocyte PPIX (figure 2). In addition, no correlation was observed between ferritin and any of the hepatic parameters.

Because of its hydrophobic nature, PPIX can only be removed from the body through the liver, where it complexes to lipids to be thereafter secreted into bile and excreted by fecal elimination [3]. Therefore, we investigated lipid metabolism (cholesterol, triglycerides, HDL and LDL) in EPP patients to evaluate if it could be modified by the level of PPIX. Results are shown in table 3: the parameters measured are all included in the interval of reference values. No correlation was noted between any of these parameters and erythrocyte PPIX.

## DISCUSSION

FECH has two substrates, iron and PPIX, and only PPIX seems to accumulate in excess in EPP. Hence, 20 to 50% of the EPP patients develop a mild anemia, the pathogenesis of which is unclear. In contrast to sideroblastic anemia [10] and congenital erythropoietic porphyria [29] which also strongly affect erythroid heme biosynthesis, the anemia resulting from FECH deficiency is not dyserythropoietic and does not induce iron overload.

The homozygous mutant mouse *Fech* $^{m/Pas}$ represents a useful model to study the pathophysiological feature of the human disease, with a residual FECH activity of 5 % in the liver and spleen, skin lesions and hepatic dysfunction with PPIX deposits [1; 4; 28]. Characterisation of erythropoiesis in these mice has revealed that FECH deficiency induces microcytic hypochromic anemia, the severity of the phenotype being strain-dependent [19]. In the present work and as previously described [14], we show that the remaining FECH activity in patients with EPP is not sufficient to maintain full erythropoiesis. Indeed, the patients present a shift towards lower values of the hematologic parameters and a defect in hemoglobinisation that seems to grow with the level of PPIX.

Strikingly, patients with EPP also present a mild thrombocytopenia, the number of platelets being inversely correlated with erythrocyte PPIX. Thrombocytopenia is a common complication of chronic liver disease, although its pathogenesis is not clear. Indeed, serum thrombopoietin has been shown to inversely correlate with liver failure of patients with chronic hepatitis [15] and cirrhosis [11], resulting in thrombocytopenia. Although we did not notice significant alteration of hepatic function in patients with EPP, one can speculate that accumulation of PPIX in the liver could interfere with the production and/or the secretion of thrombopoietin, thereby explaining at least in part the mild thrombocytopenia.

Tissue iron depletion is described as a common feature in EPP [27]. Our results further suggest that tissue iron stores are reduced in patients with EPP, as shown by the downward

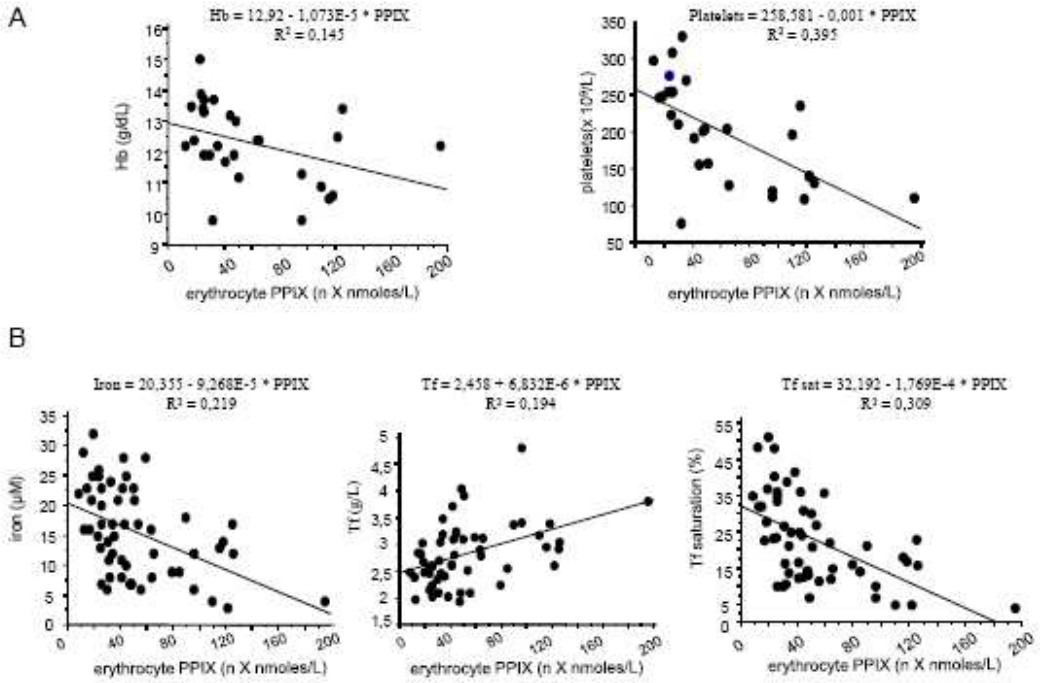


**Figure 1.** Hematologic and iron parameters correlate with erythrocyte PPIX in EPP patients.
*(A): correlation between hematologic parameters and erythrocyte PPIX in EPP patients.*
Hb concentration was evaluated in 28 patients with EPP. Platelets concentration was evaluated in 28 EPP patients and it strongly negatively correlates with erythrocytes PPIX ($r^2$ = 0,395 p = 0,0002) in these patients.
*(B): correlation between iron parameters and erythrocyte PPIX in EPP patients.*
Iron and Tf saturation were evaluated in 55 EPP patients and show a negative correlation with erythrocyte PPIX ($r^2$= 0,219 p=0,0005 and $r^2$= 0,309 p<0,0001, respectively). Tf level was evaluated in 55 EPP patients and show a positive correlation with erythrocyte PPIX ($r^2$=0,194, p=0,0006).

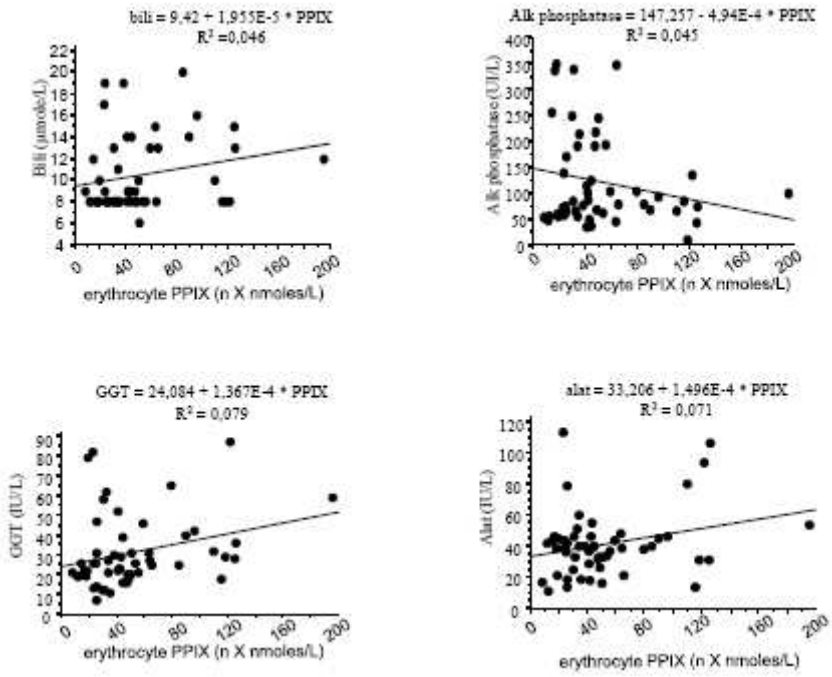


**Figure 2.** Absence of correlation between hepatic parameters and erythrocyte PPIX in EPP patients.
Hepatic parameters (alkalin phosphatase, total bilirubin, gamma GT, and ALAT [SGPT]) were were determined by standard automated methods (Dade Behring, RxL max).

**Table 3.** Hepatic and lipidic parameters in patients with EPP.

| | patients EPP | | | reference value |
|---|---|---|---|---|
| **Hepatic parameters** | all (n=50) | women (n= 24) | men (n= 26) | |
| **Alkalin phosphatase (IU/L)** | 121.1 ± 89.8 | 103.8 ± 86.5 | 137.1 ± 91.4 | < 150 |
| **Total bilirubin (µmol/L)** | 10.5 ± 3.5 | 10.3 ± 3.3 | 10.8 ± 3.7 | < 17 |
| **Gamma GT (IU/L)** | 31.2 ± 18.4 | 25.6 ± 9.7 | 36.1 ± 22.6 | < 38 |
| **SGOT/ASAT (IU/L)** | 24.2 ± 10.4 | 21.9 ± 9.6 | 26.2 ± 10.9 | < 31 |
| **SGPT/ALAT (IU/L)** | 40.9 ± 21.2 | 36.7 ± 17.7 | 44.8 ± 23.5 | < 34 |
| **Lipidic parameters** | all (n=42) | women (n= 21) | men (n= 21) | |
| **Triglycerides (mmol/L)** | 1.2 ± 0.8 | 1.0 ± 0.5 | 1.3 ± 1.0 | 0.5 – 2.0 |
| **Cholesterol * (mmol/L)** | 4.5 ± 0.8 | 4.5 ± 0.8 | 4.5 ± 0.8 | 4.6 – 6.5 |
| **Cholesterol HDL * (mmol/L)** | 1.4 ± 0.3 | 1.4 ± 0.3 | 1.4 ± 0.3 | > 0.9 |
| **Cholesterol LDL * (mmol/L)** | 2.6 ± 0.6 | 2.5 ± 0.6 | 2.6 ± 0.7 | 2.6 – 4.4 |
| **LDL/HDL *** | 1.9 ± 0.6 | 1.9 ± 0.7 | 1.9 ± 0.6 | < 3.2 |

Hepatic parameters (alkalin phosphatase, total bilirubin, gamma GT, ASAT [SGOT] and ALAT [SGPT]) were evaluated in 50 EPP patients .
Lipidic parameters (triglycerides, cholesterol [total, HDL and LDL]) were evaluated in 42 or 36 (*) EPP patients.
Data are expressed as mean ± SD.

shift of ferritin and the decrease of iron and Tf saturation with the amount of erythrocyte PPIX. Although these observations are reminiscent of what is observed in true iron-deficiency anemia [8], we could not demonstrate severe anemia in our patients but only indices of mild anemia. In addition, as previously described [14], we observed no modification in the level of sTfR in patients with EPP, suggesting that the decrease of tissue iron store is not the cause for the defect in erythropoiesis.

Interestingly, previous investigations that have described a mild anemia in patients with EPP [7; 23; 27] showed no evidence for iron loss [23]. Rather, iron has been described to be abundantly localised in erythroblasts, as membrane-bound ferritin particles, namely sideroblasts [23]. Such abnormal iron deposits are also usually observed in molecular defects of genes implicated in intramitochondrial iron use: erythroid-specific 5-aminolevulinate synthase [9] and ABCB7 [2]. In the *Fech* $^{m1Pas}$ mice, no sideroblasts were observed, but iron was shown to be redistributed from peripheral tissues to the spleen, with no loss of total body iron [19]. However, iron of patients with EPP could also be present at the site of erythropoiesis, in bone marrow. The intriguing observation of excess iron in erythroblasts of patients EPP despite low tissue iron store [23] could suggest such a redistribution of iron towards the site of erythropoiesis in EPP. Anyway, tissue iron distribution has never been extensively investigated in these patients. This is a very important issue since the existence of such redistribution of iron in the spleen would be in contradiction with the iron supplementation therapy recommended for patients with EPP that do not present additional iron loss. On the contrary, such treatment could enhance the photosensitivity of these EPP patients.

Although PPIX accumulates in erythrocytes in EPP, accumulation of iron seems to be prevented, by a mechanism that remains so far elusive. However, study of iron metabolism in *Fech* $^{m1Pas}$ mice has revealed a strong correlation between erythrocyte PPIX levels and serum Tf levels [19]. In addition, direct injection

of PPIX into these mice has been shown to directly stimulate Tf synthesis [19]. These observations suggested that PPIX may be involved in the increased of synthesis of hepatic Tf in response to insufficient iron supply for erythropoiesis, to mobilise iron from tissue stores. In the present work, we confirm this positive correlation between erythrocyte PPIX and Tf ($r^2$= 0,194 p = 0,0006) in patients with EPP. The way PPIX could influence the synthesis of hepatic Tf remains to be precised, although *in vitro* studies rather suggest an indirect pathway [19]. In addition, the limited iron accumulation in patients with EPP could result from diminished iron absorption and supply, although still matches with the limited rate of erythropoiesis. Indeed, although no alteration of the expression of the intestinal iron reductase Dcytb was noticed in the mouse model *Fech* $^{m/Pas}$ [19], one cannot rule out the possibility that FECH deficiency or PPIX accumulation itself could affect duodenal iron transport by a different pathway that remains to be determined. Indeed, the metabolite of griseofulvin (which inhibits FECH), has been shown to decrease duodenal iron transport both *in vitro* and *in vivo* [16; 17]. This study in humans strongly support previous data found in an EPP mouse model and indicates that PPIX could induce an activation of hepatic Tf synthesis. There was a correlation between erythrocyte PPIX and serum transferrin levels. These results raise the intriguing possibility that serum PPIX acts as a sensor of iron supplies to erythroid cells, signaling to the liver to stimulate transferrin synthesis when these supplies are insufficient. The precise mechanisms involved still remains to be established. In particular, considering the potential deleterious effects of iron supplementation, the investigation of body iron distribution in these patients and the precise role of PPIX in this pathway may be helpful to individualise and adapt the treatment for each EPP patient.


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