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Genetic background influences hepcidin response to iron imbalance in a mouse model of hemolytic anemia (Congenital erythropoietic porphyria)

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ABSTRACT

Clinical severity is heterogeneous among patients suffering from congenital erythropoietic porphyria (CEP) suggesting a modulation of the disease (UROS deficiency) by environmental factors and modifier genes. A KI model of CEP due to a missense mutation of UROS gene present in human has been developed on 3 congenic mouse strains (BALB/c, C57BL/6, and 129/Sv) in order to study the impact of genetic background on disease severity. To detect putative modifiers of disease expression in congenic mice, hematologic data, iron parameters, porphyrin content and tissue samples were collected.

Regenerative hemolytic anemia, a consequence of porphyrin excess in RBCs, had various expressions: 129/Sv mice were more hemolytic, BALB/c had more regenerative response to anemia, C57BL/6 were less affected. Iron status and hemolysis level were directly related: C57BL/6 and BALB/c had moderate hemolysis and active erythropoiesis able to reduce iron overload in the liver, while, 129/Sv showed an imbalance between iron release due to hemolysis and erythroid use.

The negative control of hepcidin on the ferroportin iron exporter appeared strain specific in the CEP mice models tested. Full repression of hepcidin was observed in BALB/c and 129/Sv mice, favoring parenchymal iron overload in the liver. Unchanged hepcidin levels in C57BL/6 resulted in retention of iron predominantly in reticuloendothelial tissues. These findings open the field for potential therapeutic applications in the human disease, of hepcidin agonists and iron depletion in chronic hemolytic anemia.

1. Introduction

Congenital Erythropoietic Porphyria (CEP) is a rare inborn error of heme synthesis inherited as an autosomal recessive trait. The inheritance of 2 mutant alleles for the gene encoding the enzyme Uroporphyrinogen III Synthase (UROS; *EC 4.2.1.75*) leads to excessive synthesis of type I isomers porphyrins (uroporphyrin I and coproporphyrin I), which are biologically useless. Accumulation of these pathogenic porphyrins in bone marrow erythroid cells and RBCs leads to intravascular hemolysis with massive appearance of these compounds in plasma, urine and feces [1]. CEP patients suffer from chronic hemolysis, without symptoms of ineffective erythropoiesis, and from cutaneous photosensitivity with mutilating

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involvement [2]. The clinical penetrance of CEP is heterogeneous among patients, ranging from severe hemolytic anemia, causing *hydrops fetalis* in *utero* or transfusion dependency, to mild forms restricted to skin lesions in adult life [3,4]. The mechanisms explaining the variability of CEP phenotype is still unknown but genetic (additional variants in cis or *trans*-position to the primary mutation, modifier genes), epigenetic, and/or environmental factors might be causative. As previously shown, the hemolytic phenotype in CEP patients could be modulated by sequence variations in *ALAS2,* the gene encoding the first enzyme of the heme biosynthetic pathway in erythroid cells [5].

The primary pathogenesis of hereditary and acquired hemolytic anemias is an iron overload resulting from massive outflow of RBC-free Hb and heme that are shuttled by haptoglobin and hemopexin scavengers, respectively, and cleared by spleen and liver macrophages [6]. Following activation of heme oxygenase 1 (HO-1) pathway, heme is catabolized and iron accumulates progressively in tissues. Iron overload may also derive from iron dysregulation due to reduced level of hepcidin, the iron regulatory factor produced by liver. Hepcidin downregulates gastrointestinal absorption and iron release from reticuloendothelial macrophage storage sites, leading to reduced serum iron [7]. The level of hepcidin is generally low, when the activity of erythropoiesis is stimulated [8] and its chronic repression accounts for the paradoxical condition known as iron loading anemia [9]. Erythroferrone (ERFE) has been proposed as a negative regulator of hepcidin expression, in acute anemia caused by hemorrage: the resulting renal hypoxia increases erythropoietin (EPO) production which in turn promotes the production of new red blood cells [10]. ERFE facilitates iron delivery during stress erythropoiesis but also contributes to iron overload in aemias with ineffective erythropoiesis [11].

Using a murine knock-in model of CEP, we recently showed that chronic intravascular hemolysis in CEP is associated with regenerative erythropoiesis and an efficient erythroid response in bone marrow and spleen [12]. Hepcidin was repressed and intestinal iron absorption increased. Here we aimed to expand the knowledge of variability of CEP phenotype, by examining three congenic mice strains (BALB/c, C57BL/6 and 129/Sv). The design of the project included two time points at 3 and 6 months for blood (RBC, reticulocyte, and fluorocyte counts, total porphyrins) and urine (porphyrins) analyses before sacrifice, full blood assessment (iron status hemolysis, renal and hepatic function) and tissue collection. The study highlights a multi-organ involvement related to iron overload and demonstrated that handling of chronic hemolysis has a specific iron metabolism pattern depending of mice strains, albeit hemoglobin content showed no significant difference. The evidence of iron metabolism as a potential modulating factor of the severity of the CEP phenotype opens the field of alternate therapies in the human chronic hemolytic disorders.

2. Materials and methods

2.1. Production and maintenance of congenic strains

The mouse model of CEP was initially obtained by a knock-in of the last coding exon of *Uros* gene [13] introducing a missense mutation which had been previously found in CEP patients (c.473 C > A, p.(Pro248Asn), or P248Q. Heterozygous mice (either female or male) were repeatedly back-crossed (>12 generations) to obtain genetically pure homozygotes ($Uros^{m/m}$) onto 3 different backgrounds: BALB/c, C57BL/6, and 129/Sv. The mice were maintained in the specialized animal facility of Bordeaux University, under a 12-h light/dark cycle with protective shields to avoid unwanted pain and prevent infection from cutaneous damage. They received unlimited amounts of autoclaved water and irradiated food pellets (standard laboratory mouse chow; D113; SAFE, Augy, France). Experimental procedures were performed in compliance with the French and European regulations on Animal Welfare and Public Health Service recommendations. Mice were sacrified and biological samples were collected at 3 and 6 months. No significant difference was observed between females and males (n = 6 for each gender), the data are pooled in the results section.

2.2. Analysis of blood parameters

Blood was collected by puncture of the orbital sinus on heparin- or EDTA-coated tubes. RBC, hemoglobin (Hb) and hematocrit (Ht) were measured on an animal blood counter (Scil vetABC, ABX diagnostics, Montpellier, France). Fluorocyte (porphyrin-accumulating RBCs) and reticulocyte counts were performed by flow cytometry on the FACS Canto II (BD Biosciences, France). Fluorocytes were analyzed at 550 nm (FL3-channel) with a 488 nm excitation laser; reticulocytes were labelled with orange thiazole prior to analysis at 530 ± 15 nm (FL1-channel) after 488 nm excitation.

Parameters related to iron metabolism (iron, transferrin ferritin), hemolytic process (LDH), renal and hepatic function (urea, creatinine ASAT, ALAT, alkaline phosphatase) were determined by the means of an AU400 automate (Beckman Coulter, Villepinte, France). Liquid chromatography coupled with mass spectrometry was used for mice hepcidin dosage as described previously [14].

2.3. Histological analysis

Tissue iron content was determined by acid digestion of tissue samples, followed by iron quantification on the AU400 automate [15]. For immunohistochemistry, liver sections were microwave-heated in an antigen-retrieval buffer (0.01 mol/L sodium citrate, pH 6.0) for 5 min and allowed to cool down for an additional 20 min. Slides washed with Tris buffered saline containing 0.1% Tween 20 were pretreated for 10 min with 3% H_2O_2 and incubated overnight at 4 °C with antibodies against ferroportin (FPN) (1/100, Interchim, Montluçon, France), F4/80 (1/50, Biorad, Marnes-La-Coquette), in TBST. Primary antibodies were detected with HRP–conjugated anti-rabbit antibodies (Vectastain® Universal Quick HRP Kit, CliniSciences, Nanterre, France).

2.4. Porphyrin analyses

The quantification of urinary porphyrins was performed by reverse-phase high performance liquid chromatograph with fluorimetric detection (HPLC) [16]. The quantification of porphyrins in plasma was performed by spectrofluorimetry: after excitation at 405 nm, the maximum of emission was measured at 600 nm, using uroporphyrin I as a standard [17]. Frozen tissues from kidney, liver, and spleen were mechanically dissociated in TET buffer (0.1 M Tris-Cl, 1 mM EDTA; 1% Tween 20) and lysed by successive freezing-unfreezing steps. The homogenates were extracted in methanol/HClIO4 1 M (50:50 vol) and monitored by the same fluorimetric assay as plasma samples.

2.5. Statistical evaluation

Mann-Whitney tests were used for the analysis of biochemical and hematologic data. GraphPad Prism software (San Diego California) was used for statistical analysis. The following symbols are used in Tables 1 and 2 (comparison between strains): BALB/c to C57BL/6 (*, ns), BALB/c to 129/Sv ($, ns) and C57BL/6 to 129/Sv (£, ns). In Tables 3 and 4, and Figs. 1 and 2, Mann-Whitney tests were used to compare wt and CEP within each strain. $p < 0.05$: *;

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Table 1
Porphyrin accumulation in 6 month-old CEP and wild-type (wt) mice.

		Plasma (nmol/L)	Liver (nmol/g)	Spleen (nmol/g)	Kidney (nmol/g)
BALB/c	**wt**	16.2 (1.9)	<8	<4	<1.5
	CEP	2408 (144)	604 (208)	4303 (1183)	285 (95)
C57BL/6	**wt**	12.9 (1.9)	<6	<4.5	<4.5
	CEP	2906 (727)^{ns}	410 (166)^{ns}	2517 (1071)^{ns}	384 (140)^{ns}
129/Sv	**wt**	9.2 (1.45)	<5	<10	<5.5
	CEP	6283 (1362)^{ns}	867 (399)^{ns}	1638 (739)^{ns}	644 (344)^{ns}

Data are expressed as the mean (sem) of 11–12 inbred mice.
Mann-Whitney tests were used to compare CEP mice strains. ns: not significant.

Table 2
Erythropoiesis-related parameters in 6 month-old CEP and wild-type (wt) mice.

		Hemoglobin (g/dL)	Reticulocytes (%)	Spleen weight (% body weight)	Fluorocytes (%)
BALB/c	**wt**	15.6 (0.34)	4.4 (0.2)	0.45 (0.05)	0
	CEP	8.7 (0.18) x 0.56	32.9 (1.78) x 7.48	3.79 (0.09) x 8.4	22.7 (2.23)
C57BL/6	**wt**	14.3 (0.23)	4.3 (0.3)	0.31 (0.03)	0.1
	CEP	8.9 (0.13)^{ns} x 0.58	19.8*** (0.6) x 4.60	1.43**** (0.06) x 4.6	8.4*** (0.6)
129/Sv	**wt**	14.6 (0.32)	3.4 (0.96)	0.30 (0.03)	0.1
	CEP	8.9 (0.21)^{ns} x 0.61	23.9 $^{\$\$}$ $^{£}$ (1.78) x 7.03	2.61$^{\$\$\$}$ $^{££££}$ (0.07) x 8.7	15.9$^{\$}$ $^{££}$ (1.36)

Data are expressed as the mean (sem) of 11–12 inbred mice. Mann-Whitney tests were used to compare CEP mice strains: BALB/c to C57BL/6 (*, ns), BALB/c to 129/Sv ($, ns) and C57BL/6 to 129/Sv (£, ns). $p < 0.05$: *; $p < 0.01$: **; $p < 0.001$: ***; $p < 0.0001$: ****; ns: not significant.

Table 3
Hemolysis, and iron status-related parameters in 6 month-old CEP and wt mice.

		LDH (UI/L)	Iron (μmol/L)	Transferrin Saturation (%)	Ferritin (μg/L)
BALB/c	**wt**	593 (69)	37.2 (1.6)	35.6 (1.9)	223 (11)
	CEP	2608 (310)***	35.0 (1.2)^{ns}	22.9 (0.9)***	1968 (283)****
C57BL/6	**wt**	931 (154)	20.0 (3.4)	17.5 (3.1)	388 (42)
	CEP	1926 (69)**	12.2 (1.2)*	8.3 (1.0)**	1478 (142)**
129/Sv	**wt**	873 (206)	25.2 (1.1)	21.2 (1.2)	472 (37)
	CEP	14,988 (1135)****	36.6 (2.3)**	21.1 (1.4)^{ns}	11,836 (1405)****

Data are expressed as the mean (sem) of 11–12 inbred mice. Mann-Whitney tests were used to compare wt and CEP within each strain: ns: not significant; $p < 0.05$: *; $p < 0.01$: **; $p < 0.001$: ***; $p < 0.0001$: ****.

Table 4
Liver function in 6 month-old CEP and wt mice.

		ALAT (IU/L)	ASAT (IU/L)	Alkalin phosphatase (IU/L)
BALB/C	**wt**	49.4 (9.4)	155.4 (26.7)	59.4 (5.4)
	CEP	54.6 (3.8) *	277.6 (30.2) **	75.0 (4.6) *
C57BL/6	**wt**	46.5 (6.7)	189.0 (45.3)	70.3 (10.4)
	CEP	47.1 (3.3) ^{ns}	193.0 (17.5) ^{ns}	64.4 (7.6) ^{ns}
129/Sv	**wt**	46.7 (7.4)	136.6 (20.0)	68.3 (6.0)
	CEP	100.9 (7.0)***	979.6 (42.3)****	94.0 (9.8) ^{ns}

Data are expressed as the mean (sem) of 11–12 inbred mice. Mann-Whitney tests were used to compare wt and CEP within each strain: ns: not significant; $p < 0.05$: *; $p < 0.01$: **; $p < 0.001$: ***; $p < 0.0001$: ****.

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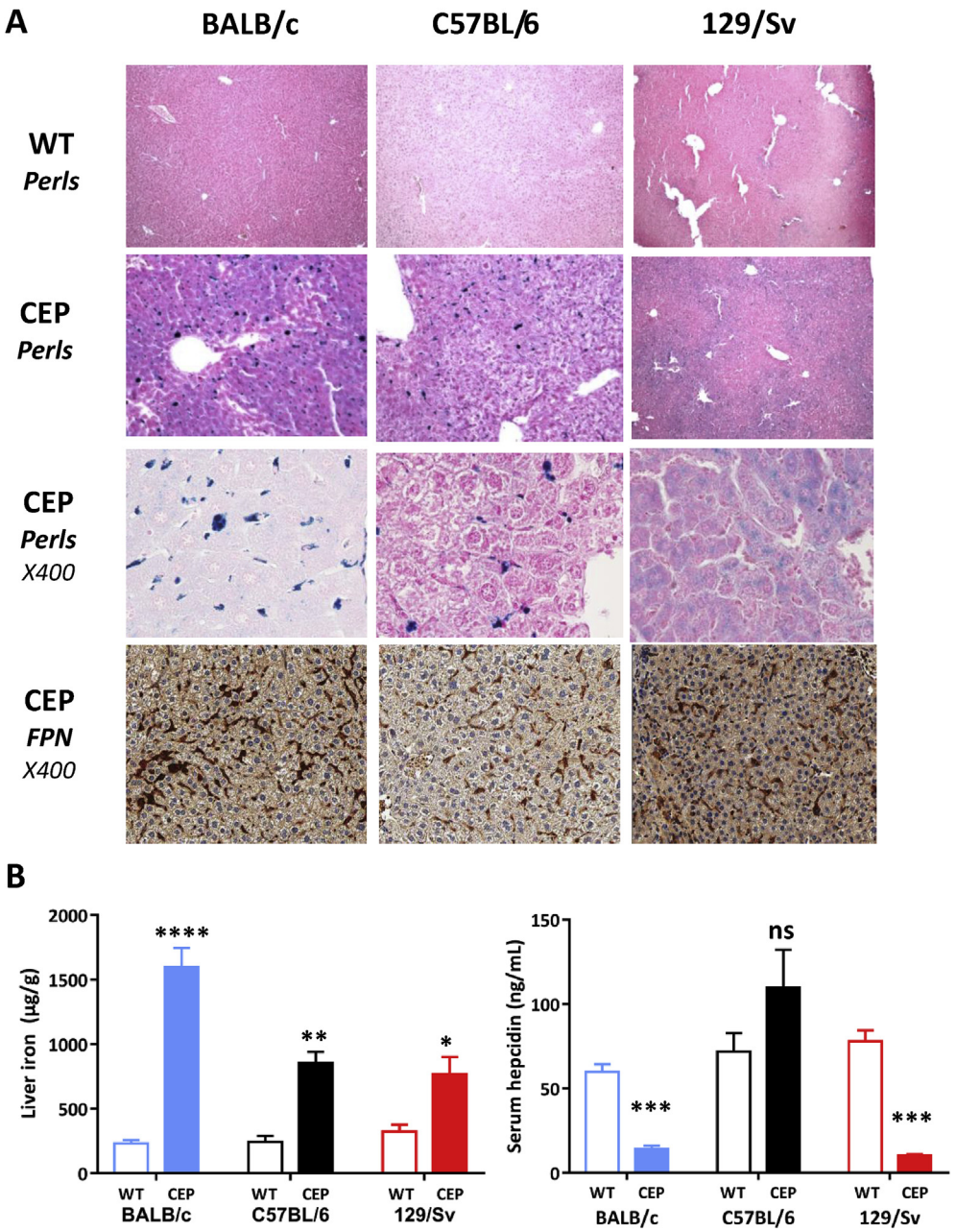

Fig. 1. Iron staining and ferroportin immunostaining related to iron content in liver slices, and serum hepcidin A - Iron distribution analyzed by Perls staining of liver slices from WT (top) and CEP mice from the BALB/c (left), C57BL/6 (middle), and 129/Sv (right) backgrounds. Magnification scale is x 10 in WT (no significant iron deposit) in the top panel and CEP 129/Sv (diffuse staining) slides, x 20 in BALB/c and C57BL/6 CEP slides, or x 400 (bottom slides). The distribution of iron deposits in CEP mice of the 3 backgrounds is exemplified by x 400 magnification of Perls stainings and immunostaining with FPN antibody (bottom). In the C57BL/6 mice, iron deposits and FPN staining were positive only in Kupffer cells with low intensity. In BALB/c and 129/Sv the positive staining was high in Kupffer cells and faint in hepatocytes. FPN-expressing Kupffer cell number was increased in BALB/c. B – Quantification of liver iron content (left panel) and serum hepcidin (right panel) in WT and CEP mice from the BALB/c, C57BL/6, and 129/Sv background. Mann-Whitney tests were used to compare wt and CEP within each strain: ns: not significant; $p < 0.05$: *; $p < 0.01$: **; $p < 0.001$: ***; $p < 0.0001$: ****.

$p < 0.01$: **; $p < 0.001$: ***; $p < 0.0001$: ****; ns: not significant. Data are expressed as the mean (sem) of 11–12 inbred mice.

3. Results

3.1. Typical hallmarks of the disease

As expected, total porphyrins were significantly increased in CEP compared to WT mice (Table 1). Porphyrin levels were consistently higher in 129/Sv mice compared to BALB/c and C57BL/6 who had similar levels, although statistical tests did not reach significance due to a large variability. All mice had massive accumulation of porphyrins in spleen, liver, kidney and urine.

3.2. Identical severity of anemia associated with varying degrees of hemolysis

Erythropoiesis activity was evaluated by measuring hemoglobin and reticulocyte counts at 3 and 6 months (Table S1 and 2). Mean hemoglobin levels were comparable in each strain at 3 and 6 months: the range was 8.7–8.9 in CEP versus 14.3–15.6 g/dL in 6-month-old wt mice. However, reticulocyte counts differed at 3

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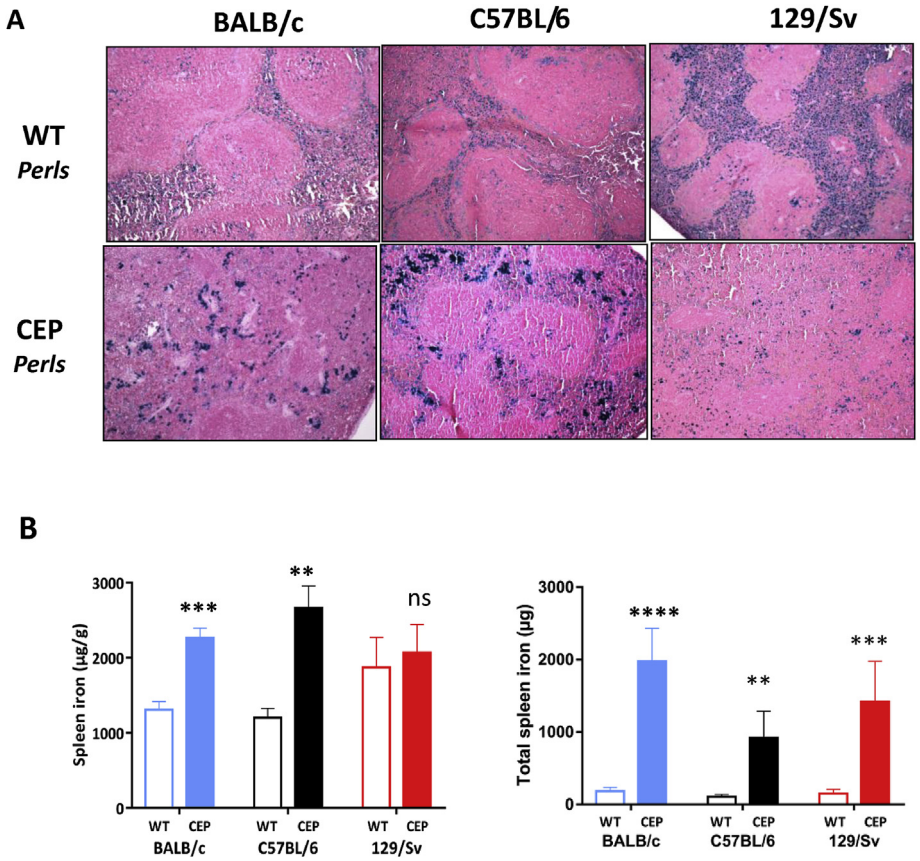

Fig. 2. Iron staining and iron content in spleen slices A - Iron distribution was analyzed by Perls staining of spleen sections from WT (top) and CEP (bottom) affected mice from the BALB/c (left), C57BL/6 (middle), and 129/Sv (right) background. Magnification scale is x 10 in all panels. B - Quantification of spleen iron content in WT and CEP mice from the BALB/c, C57BL/6, and 129/Sv background. Mann-Whitney tests were performed as above. The mean content per weight unit of tissue is shown in the left panel (µg/g as above). As splenomegaly was manifest in CEP mice, total iron content (µg) is shown in the right panel. ns: not significant; $p < 0.01$: **; $p < 0.001$: ***; $p < 0.0001$: ****.

months, and strain differences were more pronounced at 6 months (23.9:129/Sv, 32.9:BALB/c, 19.8%:C57BL/6). Fluorocyte count, which reflects the accumulation of porphyrin in reticulocytes [3], differed in mice aged 3 months and reached statistical significance at 6 months with similar distribution among strains (15.9:129/Sv, 22.7:BALB/c, 8.4%:57BL/6). Splenomegaly, due to chronic hemolysis, was severe in 129/Sv and BALB/c (8.4 and 8.7-fold) or moderate in C57BL/6 mice (4.6-fold the wt size). Increased serum LDH levels (Table 3) revealed marked hemolysis, mostly in 129/Sv (14,998 UI/L, 20-fold) compared to BALB/c (2608 UI/L, 4-fold) and C57BL/6 (1926 UI/L, 2-fold). The more severe hemolysis in 129/Sv CEP mice was also evidenced by a preferential increase of ASAT versus ALAT (7-fold vs 2-fold) (Table 4).

These data indicate that C57BL/6 exhibited a mild hemolysis phenotype compared to 129/Sv and BALB/c strains, with identical hemoglobin levels.

3.3. Specific iron-overload patterns

Iron status was assessed from serum parameters (Fe, transferrin, transferrin saturation rate, ferritin, hepcidin) and iron accumulation was evaluated by both tissue iron content measurement and Perl's staining. Hyperferritinemia was more pronounced in 129/Sv compared to BALB/c and C57BL/6 strains (Table 3).

Iron deposits had differential patterns among the 3 backgrounds in the liver and spleen. In the liver of 129/Sv mice, Perl's staining showed iron accumulation predominantly in hepatocytes, only few macrophages were Perl's positive (Fig. 1A). Iron overload was obvious in Kupffer and endothelial cells of liver sections derived from C57BL/6. In BALB/c mice, hepatocytes, Kupffer and endothelial cells were all Perl's positive with predominance of iron deposit in Kupffer cells (Fig. 1A). In addition, immunostaining of Kupffer cells with specific anti-F4/80 antibody (data not shown) showed an increased number of these cells in BALB/c versus both other strains, which may contribute to the high rate of iron accumulation. Hepatic iron content was increased in all strains, most significant in BALB/c mice (Fig. 1B). No change in liver size was noted in each CEP strain.

In the spleen of 129/Sv mice, despite the high level of hemolysis, the reticulo-endothelial cells were not iron overloaded compared to C57BL/6 and BALB/c mice (Fig. 2A). Iron content in spleen reflected iron deposition in the 3 strains with increased amount in C57BL/6 and BALB/c but no apparent excess accumulation in 129/Sv CEP mice strain (Fig. 2B left panel). However spleen size was enlarged in CEP mice, 9.5, 4.2, and 8.7-fold, in BALB/c, C57BL/6 and 129/Sv, respectively, and a significant accumulation of iron was present in 129/Sv mice, considering global iron loading (Fig. 2B right panel).

3.4. Specific features of hepcidin/ferroportin axis

Measurements of serum hepcidin showed full repression of hepcidin secretion in BALB/c and 129/Sv, but no significant change in C57BL/6 CEP mice (Fig. 1B). Since hepcidin acts primarily on the iron exporter ferroportin (FPN) by internalization and degradation of the protein, we analyzed FPN expression in liver by

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immunostaining and found a lower labelling in C57BL/6 strain compared to BALB/c and 129/Sv (Fig. 1A). In C57BL/6 mice, only Kupffer cells exhibited positive, although moderate, FPN staining, while in 129/Sv both cell types Kupffer and hepatocytes were strongly stained. Of note, serum ferritin and transferrin saturation were significantly decreased in C57BL/6 compared to the other strains (Table 3).

3.5. Specific response to a hemolytic challenge in wild-type mice

Finally, we wondered wether the 3 strains would have different responses to hemolysis which may explain the difference in hepcidin repression. We performed an acute hemolytic challenge with 100 mg/kg phenothiazine (PHZ) injection in WT mice and then monitored serum hepcidin: its repression occurred earlier in 129/Sv (day 2) than in BALB/c (day 3) mice. No change was observed 3 days post-PHZ injection in C57BL/6 mice suggesting a higher resistance to the hemolytic process in this strain (Fig. 3).

4. Discussion

The initial purpose of the study was the description of a quantitative trait explaining a significant part of the phenotypic variability commonly described in CEP patients.

The genetic basis of UROS deficiency has been well characterized with the identification of about forty different mutations in the *UROS* gene [4,18]. Genetic heterogeneity in CEP has been recently exemplified by the description of *ALAS2* and *GATA-1 trans*-acting variants which influence disease expression [5,19].

In Human, the severity of the phenotype in patients harboring the same molecular defect in *UROS* gene is related to the level of porphyrin accumulation in RBCs and urine [3,20]. Porphyrin accumulation is the consequence of the excessive stimulation of the erythropoietic heme pathway, triggered by heme deficiency, which exacerbates the production of hydroxymethylbilane (HMB), spontaneously converted to the uroporphyrinogen I isomer. Then, its oxidized form, uroporphyrin I (URO I) is a photo reactive product and a dead-end pathway. URO I accumulation in erythroid cells, mainly reticulocytes, results in premature death and increased turn-over of RBCs, which are ultimately trapped by reticulo-endothelial cells, residing in spleen and liver. As a water-soluble molecule, URO I is steadily eliminated in urine, albeit the mechanisms of its clearance through the nephron have not been thoroughly studied.

The strains used in this study reproduced the main aspects of the human pathology including the erythroid dysfunction, chronic hemolysis, and the cutaneous UV photosensitivity. We choose to limit cutaneous lesions by the use of constant photoprotection through filtering covers, because the presence of the fur renders the model less relevant for comparison with human skin, regarding UV exposure. The study was then conducted towards the erythroid features of the disease, which represent the main life-threatening line in the human disease. As genetic backgrounds we choose 129/Sv strain that is commonly used for implantation in homologous recombination experiments using ES cells, C57BL/6 strain, used to facilitate chimera selection from coat color, and BALB/c strain that is largely used in gene transfer experiments and thoroughly characterized in this field in our hands [12,21,22].

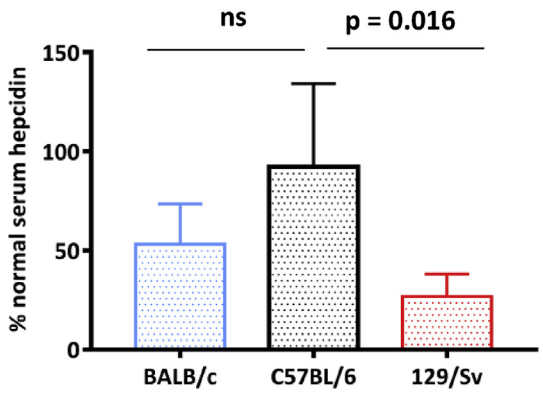

Fig. 3. Hepcidine response to a hemolytic challenge in wt miceSerum hepcidin was measured in wild-type animals of each mice strain, two days after the injection of the hemolyzing agent Phenylhydrazin (100 mg/kg). The hepcidin ratio is expressed as the percentage of basal value (saline injected mice), for each lineage.

The main quantitative trait characterizing CEP, porphyrin excess in plasma and urine or tissue, showed large variability between the strains, so that subtle changes were totally masked. Unexpected findings indicated that: 1- the chronic hemolysis differs between the strains and 2- chronic iron overload, resulting from hemolysis, has specific patterns linked to hepcidin production levels. In CEP mice, the impact of hemolytic anemia on hepcidin synthesis and iron metabolism has been thoroughly documented in the BALB/c background by previous studies [12]. Actually, the comparison of three mouse strains, including BALB/C as a reference, raised physiopathological points which pave the way for future investigations. C57BL/6 and 129/Sv exhibited two extreme phenotypes of CEP disease from the hematological point of view. The high sensitivity to hemolysis of 129/Sv strain seems to be related to high production of pathogenic porphyrins and accumulation in RBCs and reticulocytes. However, despite different degrees of hemolysis, all strains exhibited similar degrees of anemia (equal hemoglobin levels). Anemia in CEP patients is usually attributed to intravascular hemolysis [1,4], while our data on mouse models postulate additional mechanisms contributing to the anemia and highlight potential multi-organ involvement related to iron overload.

In C57BL/6, unlike the other strains, the erythropoietic regeneration was moderate and not associated with a repression of systemic hepcidin. Normal level of hepcidin may account for iron retention in macrophages as illustrated by liver and spleen Perls staining, decreased ferroportin expression and low transferrin saturation rate. This lack of hepcidin repression protects parenchymal cells from iron overload and may limit erythropoiesis regeneration by restricting iron availability to erythroid cells. It has been already shown that increased level of hepcidin in thalassemic mice (th3/+) overexpressing Hamp1 exhibited not only reduced organ iron overload but also decreased formation of insoluble membrane-bound globins and reactive oxygen species, which improved the anemia and reduced the splenomegaly [23]. Beneficial effects of increased hepcidin level in C57BL/6 CEP mice may also be exemplified by the obvious reduction of hemolysis and spleen size. ALAS2 mRNA contains an iron responsive element (IRE) in the untranslated region [24]. During low iron availability, IRE-binding proteins bind to this IRE, block translation, and diminish ALAS2 expression and activity, which attenuates porphyrin biosynthesis [25]. Recently, iron deprivation was shown to improve the photosensitivity and hemolysis symptoms in a CEP patient [26]. Thus, a reduction of erythroid iron availability, by increased hepcidinemia, could downregulate the proximal step of heme assembly, and subsequently, limit the accumulation of toxic porphyrins. Therefore, therapeutic strategies using hepcidin agonists may also have benefit in CEP. Indeed, hepcidin agonists were shown to limit iron burden in several iron loading anemias, including sideroblastic anemias and thalassemias [27–29].

In 129/Sv mice, hemolysis was profound and the resulting erythroid expansion minimized serum hepcidin level. As a consequence, massive parenchymal overload occurred, favored by the

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emptied macrophage compartment. The normal rate of transferrin saturation reflects the huge consumption of iron by the highly active regenerative erythropoiesis. Thus hepcidin repression, related to the high erythropoietic activity led to porphyrin accumulation in RBC and maintained the vicious circle of hemolysis. In addition, hepcidin repression in 129/Sv seems to occur independently from porphyrins accumulation, since PHZ-induced acute hemolysis was sufficient to cause hepcidin decline in129/Sv and Balb/C mice, while C57BL/6 escaped this repression, suggesting strain-dependent regulation of hepcidin. ERFE is known as the main repressor of hepcidin expression, controlled by EPO, as shown in ERFE KO mice [10] and mouse models of anemias with ineffective erythropoiesis [30]. In a previous study, we evidenced an increased expression of ERFE mRNA in bone marrow cells of CEP BALB/c mice [12]. Erfe mRNA levels might differ among the mice strains in study, thus explaining various down-regulatory effects on hepcidin. Unfortunately, we could not test the hypothesis at the time of sample collection, since a commercialized assay for mice Erfe was not yet available.

Modifier genes in iron metabolism were already specifically described in C57BL/6, a strain frequently used in loci mapping studies [31]. A *Mon1a* gene variant, found solely in C57BL/6 strain, was suggested to explain a moderate phenotype of hemochromatosis, given that Mon1a is involved in the membrane trafficking of proteins including ferroportin [32]. In the β-thalassemia intermedia model ($Hbb^{th1/th1}$) in the same C57BL/6 strain, hepcidin repression was absent, despite highly active ineffective erythropoiesis [33], in keeping with the less severe iron overlaod seen in this study.

To conclude, this model of chronic hemolysis indicated that iron metabolism itself and secondary iron overload may contribute to explain subtile changes in CEP phenotype in mice and humans. The study illustrated the involvement of hepcidin in heme biosynthesis and porphyrin production by erythroid cells, thus highlighting the therapeutic potential use of hepcidin and/or iron chelation in CEP and possibly in other erythropoietic porphyrias and chronic hemolytic disorders.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.09.141.

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