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Caroline Schmitt, Hugo Lenglet, Angèle Yu, Constance Delaby, Arndt Benecke, Thibaud Lefebvre, Philippe Letteron, Valérie Paradis, Staffan Wahlin, Sverre Sandberg, et al.

### ► **To cite this version:**

Caroline Schmitt, Hugo Lenglet, Angèle Yu, Constance Delaby, Arndt Benecke, et al.. Recurrent attacks of acute hepatic porphyria: major role of the chronic inflammatory response in the liver. *Journal of Internal Medicine*, 2018, 284 (1), pp.78-91. 10.1111/joim.12750 . hal-02503870

**HAL Id: hal-02503870**

**<https://hal.science/hal-02503870>**

Submitted on 10 Mar 2020

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**R1****TITLE**

**Recurrent attacks of acute hepatic porphyria: major role of the chronic inflammatory response in the liver**

**SHORT TITLE**

**Recurrent attacks of acute intermittent porphyria**

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## ABSTRACT

**Background.** Acute intermittent porphyria (AIP) is an inherited disorder of heme metabolism characterized by life-threatening acute neurovisceral attacks due to the induction of hepatic  $\delta$ -aminolevulinic acid synthase 1 (ALAS1) associated with hydroxymethylbilane synthase (HMBS) deficiency. So far, the treatment of choice is hemin which represses ALAS1. The main issue in the medical care of AIP patients is the occurrence of debilitating recurrent attacks.

**Objective.** The aim of this study was to determine whether chronic hemin administration contributes to the recurrence of acute attacks.

**Methods.** A follow-up study was conducted between 1974 and 2015 and included 602 French AIP patients, of whom 46 had recurrent AIP. Moreover, we studied the hepatic transcriptome, serum proteome, liver macrophage polarization and oxidative and inflammatory profiles of *Hmbs*<sup>-/-</sup> mice chronically treated by hemin and extended the investigations to 5 explanted livers from recurrent AIP patients.

**Results.** The introduction of hemin into the pharmacopeia has coincided with a 4.4-fold increase in the prevalence of chronic patients. Moreover, we showed that both in animal model and human liver, frequent hemin infusions generate a chronic inflammatory hepatic disease which induces HO1 remotely to hemin treatment and maintains a high ALAS1 level responsible for recurrence.

**Conclusion.** Altogether this study has important impacts on AIP care underlying that hemin needs to be restricted to severe neurovisceral crisis and suggests that alternative treatment targeting the liver such as ALAS1 and HO1 inhibitors, and anti-inflammatory therapies should be considered in patients with recurrent AIP.

## KEYWORDS

Acute intermittent porphyria / Heme oxygenase 1 / Hemin / Inflammation / Iron overload

## INTRODUCTION

Acute intermittent porphyria (AIP), the most common acute hepatic porphyria, is an autosomal dominant disease that occurs as a result of a 50% decrease in the activity of hydroxymethylbilane synthase (HMBS), the third enzyme of the heme biosynthesis pathway [1]. AIP is characterized by the intermittent occurrence of neurovisceral attacks, which typically consist of severe abdominal pain and may include hypertension, tachycardia, confusion, motor paralysis and seizures and may be life-threatening. AIP is estimated to affect 5.4 per million people in European countries [2]. The pathogenesis of the acute attacks is hypothesized to result from an overproduction of a hepatic neurotoxic metabolite, presumably  $\delta$ -aminolevulinic acid (ALA) [3, 4]. This notion is consistent with the correction of the disease by liver transplantation in patients with severe AIP [5-9]. Moreover, when domino liver transplantations were performed, the recipients developed biological and clinical acute porphyric attacks [10]. Altogether, these observations strongly support the hypothesis that the liver is the main organ involved in the pathogenesis of acute attacks.

The induction of the acute attacks is related to environmental or hormonal factors, such as cytochrome P450-inducing drugs, fasting, hormonal fluctuations during the menstrual cycle, infection or inflammation. These factors either directly induce the first enzyme of heme biosynthesis,  $\delta$ -aminolevulinic acid synthase 1 (ALAS1), or increase the demand for heme synthesis in the liver and subsequently suppress the negative feedback of ALAS1. Carbohydrate loading, usually with intravenous glucose, may be an effective treatment in patients with non-complicated acute attacks (without peripheral and central neurological signs or hyponatremia). Glucose administration, together with the resulting secondary increase in insulin, represses *ALAS1* transcription via the inhibition of the transcriptional coactivator PGC-1 $\alpha$  [11]. However, intravenous human hemin administration, which restores the free

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3 heme pool and the negative feedback of ALAS1, is, so far, the treatment of choice of acute  
4 attacks of porphyria. Early human hemin infusion is a highly effective treatment [12].  
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9 AIP is a low penetrance disorder. The penetrance has been estimated to be 23% in European  
10 countries [2]. Most symptomatic AIP patients experience a limited number of acute attacks in  
11 a lifetime, the attacks often occur once and in all cases the attacks are associated with clearly  
12 identified precipitating factors. However, a limited but significant number of patients become  
13 disabled by repetitive acute episodes with or without concomitant precipitating factors. These  
14 patients are treated with repeated heme infusions either to treat or to prevent acute attacks [13-  
15 17]. Some of these patients will even require monthly to weekly heme infusions. In Europe,  
16 approximately 5% of AIP patients suffer from recurrent attacks [2]. In addition to recurrent  
17 acute attacks, these chronic patients encounter many medical side effects and have markedly  
18 impaired quality of life [18]. The large majority of these patients respond well to heme  
19 therapy, but long-term treatment may induce a dependency on exogenous heme, a need for  
20 indwelling access due to alterations in the superficial venous system, a rare but severe  
21 thrombotic event [15, 16].  
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In the current study, we followed up a cohort of 602 symptomatic patients, of whom 46 had  
recurrent AIP, in order to document the natural history of the occurrence of chronicity in AIP.  
To elucidate the role of hemin in the recurrence of AIP attacks, we studied the hepatic  
transcriptome, serum proteome, liver macrophage polarization and oxidative and  
inflammatory profiles of an AIP mouse model which reproduces recurrent neurovisceral crisis  
treated by hemin. Moreover, thanks to the European Porphyria Network (EPNET), we  
extended the investigations to 5 human liver explants. We showed that chronic heme  
administration induces liver iron overload and sustains a strong oxidative stress response,

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3 which is known to activate HO1. Surprisingly, in the AIP mouse model and human liver  
4 explants, we demonstrate that regular hemin administration induces chronic hepatic  
5 inflammation responsible of a heme oxygenase 1 (HO1) induction remotely to heme  
6 administration that triggers ALAS1 and thus recurrence.  
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## MATERIALS AND METHODS

### Animal experiments

All animal experiments were performed according to procedures approved by the Bichat-Debré ethics committee. Animals were housed in a controlled environment with a 12 h light-dark cycle with free access to water and food. Animals were female C57BL/6 *Hmbs*<sup>-/-</sup> mice [19], 8 weeks old, weighing 20-25 g. Drugs (phenobarbital 100 mg/kg, heme arginate 8 mg/kg) were injected intraperitoneally. For each group, we treated 6 females (Figure 2A). Every week, we treated the mice with phenobarbital for 3 days followed by a 2-day treatment with hemin (PB/HA group) or excipients (arginine, ethanol and propylene glycol) (PB/Excipients group) or saline (PB/Saline group). These treatments were repeated for 8 weeks. Another control group consisted of a non-induced group of mice injected every day with saline (Saline group). When animals were sacrificed, liver tissues and blood samples were harvested.

### Microarray analysis of gene expression in the mouse liver

The mouse liver transcriptome was analyzed using Mouse Genome Survey Arrays v2.0 (Applied Biosystems, Foster City, CA, USA), containing 32,996 probes (60-mer oligonucleotide), which represented a set of 32,381 annotated mouse genes. A total of 12 independent experiments were performed for the two different biological conditions (PB/HA and PB/Saline groups). Transcriptome statistical analyses were performed similarly to those already described [20, 21].

### Proteome study of mouse plasma

Two-dimensional electrophoresis (2-DE) was carried out on plasma samples using the IPGphor Isoelectric Focusing System and the Ettan dalt six electrophoresis system

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3 (Amersham Biosciences, Little Chalfont, UK). Images of gels were digitized with a calibrated  
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5 densitometer (GS-800, Bio-Rad, Hercules, CA, USA). Proteins of interest were manually  
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7 excised and digested in-gel. Peptides were then identified either by peptide mass  
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9 fingerprinting (MALDI-TOF MS, Voyager DE-PRO in reflectron mode, Applied Biosystems,  
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11 Foster City, CA, USA) or by peptide sequencing using nano(n)ESI-IT MS/MS (Finnigan  
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13 LCQ IT mass spectrometer, ThermoQuest, Finnigan MAT, San Jose, CA, USA).  
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### 16 17 18 **Cytokine determination in mouse liver samples**

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20 After preparation, the levels of 10 mouse cytokines and chemokines in liver homogenates  
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22 were simultaneously analyzed using the MILLIPLEX® MAP Mouse Cytokine/Chemokine  
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24 Magnetic Bead Panel (Millipore, Billerica, MA, USA), according to the manufacturer's  
25  
26 instructions. The 10 cytokines and chemokines that were analyzed included Ifn- $\gamma$ , Il-1 $\beta$ , Il-4,  
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28 Il-6, Il-10, Il-12 (p40), Il-13, monocyte chemoattractant protein 1 (Mcp-1), macrophage  
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30 inflammatory protein 1 $\alpha$  (Mip-1 $\alpha$ ) and Tnf- $\alpha$ .  
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### 35 **Myeloperoxidase and leukotriene measurement in the mouse liver**

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37 Mouse liver myeloperoxidase concentration was measured via ELISA (HK210, Hycult®  
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39 Biotech, Uden, Netherlands). Leukotriene E4 is a product of the 5-lipoxygenase pathway in  
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41 activated mast cells, eosinophils, and monocytes. The concentrations were determined in the  
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43 mouse livers with a commercially available enzyme immunoassay kit, the Leukotriene E4  
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45 EIA Kit (Cayman Chemical, Ann Arbor, MI, USA).  
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### 50 **Oxidative stress analysis of the mouse liver**

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52 The concentration of 8-isoprostane, a marker of lipid peroxidation, was determined in the  
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54 mouse liver samples with a commercially available enzyme immunoassay kit, the 8-  
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3 Isoprostane ELISA Kit (Cayman Chemical, Ann Arbor, MI, USA). The levels of reduced  
4 glutathione (GSH) and oxidized glutathione (GSSG) were measured via capillary  
5 electrophoresis using the CEofix™GSH kit (Analis, Suarlée, Belgium) according to the  
6 manufacturer's recommendations and the P/ACE™ MDQ Capillary Electrophoresis system  
7 (Beckman Coulter, Brea, CA, USA). GSH/GSSG ratios were normalized according to the  
8 weight of the liver in each sample.  
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### 18 **French cohort of AIP patients**

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20 Between 1974 and 2015, 602 symptomatic AIP patients were diagnosed at the French Center  
21 of Porphyrria. The criteria for AIP diagnosis followed the EPNET guidelines. All patients  
22 presented with at least one acute attack that was biochemically confirmed by the presence of a  
23 massive excretion of urinary ALA and porphobilinogen (PBG). The porphyrin profile in  
24 urine, feces and plasma samples allowed a differential diagnosis, eliminating both variegate  
25 porphyria and hereditary coproporphyrria. The diagnosis was then confirmed by the presence  
26 of a 50% decrease in HMBS activity in erythrocytes and/or was complemented by the  
27 identification of a causative mutation in the *HMBS* gene via sequencing. From 1974 until  
28 2015, a total of 46 patients were chronically affected by acute attacks and were defined as  
29 recurrent AIP patients according to the EPNET classification (4 or more acute attacks for one  
30 or more years) [2]. These 46 patients required repeated IV infusions of human hemin either to  
31 treat or to prevent the occurrence of acute attacks.  
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### 48 **Human liver samples**

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50 All procedures were performed in accordance with the 1983 and 2008 revisions of the  
51 Declaration of Helsinki, and the study was approved by the Institutional Review Board and  
52 the Hospital Ethics Committee of the Bichat University Hospital. Small pieces of liver from  
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3 five AIP subjects from France, Sweden and Norway were obtained (Table S1). These 5  
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5 patients underwent orthotopic liver transplantation (OLT) due to the occurrence of recurrent  
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7 and debilitating attacks that were responsible for a major deterioration in quality of life.  
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9 Following OLT, urinary ALA and PBG levels rapidly normalized, and the attacks  
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11 immediately stopped. Control liver tissues were obtained at the border of liver adenoma  
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13 tumors from 10 non AIP subjects.  
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18 More details and more materials and methods are provided in the supplementary materials.  
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## RESULTS

### **Natural history of AIP in France since 1974**

Since the establishment of the French Center of Porphyria in 1974 and through 2015, 602 symptomatic AIP patients with at least one acute attack of porphyria were diagnosed (of whom 81% were female). A total of 88% of patients experienced a single acute crisis, 4.4% exhibited an intermittent crisis (between 1 and 3 acute attacks/year), and 7.6% (46 patients) were disabled by repetitive acute episodes ( $\geq 4$  attacks/year), most of which occurred without obvious precipitating factors.

### **The number of AIP patients with recurrent acute attacks increased following the introduction of heme into the pharmacopeia**

In France, the administration of human hemin started in 1986. The number of AIP patients with recurrent attacks increased from 1.7% in 1985 (4 recurrent AIP patients from a total of 230 AIP patients) before the introduction of hemin to 7.5% in 2008 (40 recurrent AIP patients from a total of 536 AIP patients). The ratio of recurrent patients has now stabilized to 7.6% (46 recurrent AIP patients from a total of 602 AIP patients) (Figure 1). Recurrent AIP patients already existed before the introduction of human hemin, but after its introduction, the frequency of recurrent AIP increased by 4.4-fold, and the interval between crises shortened, in some cases to less than one week. Concerning the evolution of the 46 patients, 32 still exhibit recurrent AIP, of whom 18 patients receive human hemin as a preventive treatment every 7 to 14 days (before the appearance of any clinical symptoms), and most of them take oral opiate medication for daily pain treatment. In rare cases ( $n=6$ ), it has been possible to obtain complete cessation of human hemin treatment. Moreover, 5 patients underwent orthotopic liver transplantation (OLT) due to the presence of permanent pain and a poor quality of life (one patient died). Complete biochemical resolution was observed in the 5 patients after

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3 transplantation, and the 4 patients who are still living have remained attack-free up to now (3-  
4 to 8-years post-transplantation). On the 46 patients, a total of 3 patients died from unrelated  
5 diseases.  
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### 10 11 **AIP mouse model and long-term treatment with hemin**

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13 To study the hepatic consequences of the chronic administration of hemin, we studied the  
14 *Hmbs*<sup>-/-</sup> mouse model, which clinically develop a peripheral neuropathy as observed in some  
15 AIP patients [19, 22]. In this model, intraperitoneal injections of phenobarbital induced *Alas1*  
16 expression, resulting in a massive but transient overproduction of ALA and PBG without  
17 typical neurovisceral symptoms [19].  
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26 To reproduce recurrent attacks with frequent administration of human hemin, we set up a  
27 protocol of a chronic induction of an AIP crisis treated by hemin in the AIP mouse model  
28 (Figure 2A). PBG level was measured in urines at weeks 1, 3, 6 and 8 (Figure 2B). After the  
29 phenobarbital injection, the concentration of PBG increased and rapidly returned back to  
30 normal, as already described [23]. To further validate the biochemical induction of *Alas1* in  
31 our model, we analyzed the liver expression of *Alas1* using western-blot analysis. When mice  
32 were sacrificed after the last phenobarbital injection on day 3 of the eighth week (PB/Saline  
33 D3 group), we observed a 15-fold over-expression of *Alas1* when compared to non-induced  
34 mice (Saline group, Figure 2C).  
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### 48 **Paradoxical *Alas1* induction after chronic hemin infusion in the liver of *Hmbs*<sup>-/-</sup> mice**

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50 We analyzed the protein expression of *Alas1* in the liver of all groups of mice sacrificed at  
51 day 5 of the eighth week. As shown in Figure 2C, 2 days after the last phenobarbital injection  
52 (PB/Saline group), *Alas1* expression returned to the basal state, but surprisingly, after  
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3 repeated heme treatment (PB/HA group), *Alas1* expression in the liver was 3-fold higher than  
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5 in non-induced (Saline group) and saline treated mice (PB/Saline group).  
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### 8 9 **Repeated hemin injection activated HO1 in the liver of *Hmbs*<sup>-/-</sup> mice**

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11 Over-expression of the *HO1* gene was observed in the liver in the heme arginate treated group  
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13 compared to all other groups (Figure 2D). A 9-fold induction was identified with microarrays,  
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15 and an 11-fold increase was detected via quantitative RT-PCR, probably in response to the  
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17 high cumulative dose of hemin. As shown by western-blot analysis, HO1 protein expression  
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19 was also highly increased in the livers of mice treated with heme arginate compared to all  
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21 other groups (Figure 2E).  
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### 26 27 **Hepatic genome-wide transcriptomic modulation after chronic heme arginate infusion** 28 29 **in *Hmbs*<sup>-/-</sup> mice**

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31 The liver transcriptome of two groups of *Hmbs*<sup>-/-</sup> mice was analyzed. Each group (n=6) was  
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33 treated sequentially for 8 weeks with phenobarbital and either treated with heme arginate  
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35 (PB/HA group) or saline (PB/Saline group). One hundred and fourteen probes were shown to  
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37 be modulated by hemin treatment when using a *P*-value threshold of 0.1. Among these 114  
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39 probes, 43 transcripts were down-regulated, and 50 were up-regulated (Table S2). The extent  
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41 of single probe modulation ranged from a fold-change of 0.01 to 29.86. To interpret the  
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43 biological significance of global gene modulation, we carried out functional enrichment  
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45 analysis implemented by g:Profiler software [24]. Among the 9 biological processes identified  
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47 *in silico*, 4 were significantly enriched in genes belonging to biological pathways linked to  
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49 inflammation (Table S3). Interestingly, several genes involved in reactive oxygen species  
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51 metabolism were part of the transcriptomic picture of chronic heme administration, including  
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54 i) *Cybb*, which encodes a component of the phagocyte oxidase enzyme complex, ii) *Rac2*,  
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3 which augments the production of reactive oxygen species (ROS) by NADPH oxidase and iii)  
4 glutathione S-transferase genes, which include genes such as *Gstm3*, *Gstm4* and *Gstp1*. In  
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7 addition, genes in the Bmp6-Id1 pathway controlling liver Hpcidin transcription were also  
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9 induced. Altogether, these data of liver transcriptome showed that chronic heme infusions in  
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11 the mouse model significantly modified biological pathways linked to inflammation,  
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13 oxidative stress and iron metabolism.  
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### 18 **Repeated heme arginate infusions induce inflammation and oxidative stress in the liver** 19 **of *Hmbs*<sup>-/-</sup> mice**

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21 We further analyzed a set of inflammatory and ROS markers in liver extract either at the  
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23 mRNA or protein level. The expression of inflammatory cytokines was elevated in the liver of  
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25 the mice treated by heme arginate (Table 1). The myeloperoxidase (Mpo) level was markedly  
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27 increased in the heme arginate group (5.9-fold; Figure 3A), which suggests a polynuclear  
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29 neutrophil infiltration in the liver. A high leukotriene level was also observed in the liver  
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31 (11.8-fold in the heme arginate group; Figure 3A). Markers of oxidative stress were also  
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33 measured in the liver of all groups of mice. In the heme arginate group, the reduced-oxidized  
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35 glutathione ratio was decreased, and the level of 8-isoprostane was increased (0.3- and 12.9-  
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37 fold, respectively; Figure 3B) compared to the PB/Saline group.  
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44 To investigate the effect of chronic heme infusions at the systemic level, we analyzed the  
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46 plasma proteome in mice from the heme arginate group compared to the PB/Saline group.  
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48 The analysis revealed that 11 plasma proteins were differentially expressed (Table S4),  
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50 among which 4 were acute phase proteins either up-regulated (Serum amyloid P component,  
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52 Complement C3 and Haptoglobin) or down-regulated (Transthyretin).  
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### Repeated heme arginate infusions induce iron overload in the liver of *Hmbs*<sup>-/-</sup> mice

Repeated hemin injections and its degradation by HO1 release large amounts of iron. By measuring the liver iron content in treated mice, we showed a modest but significantly higher iron content than in non-hemin treated mice (Figure S1A). The liver transcription profile of the mice also showed an over-expression of 2 genes in the heme arginate group, *Bmp6* and *Id1*, which were first identified by microarray analysis and subsequently confirmed by qRT-PCR (Figure S1B). *Bmp6* and *Id1* genes were described to belong to the same pathway of hepcidin regulation [25]. This pathway is activated in response to iron overload [25].

### Metabolic changes in the liver of recurrent AIP patients who receive frequent hemin administration

Explanted livers from 5 recurrently afflicted AIP patients were studied. All 5 patients received a large amount of human hemin before transplantation at a frequency ranging from once a week to once a month. The last infusion before OLT took place 3 hours before in two patients (patients P4 and P5), approximately 24 h before for one patient (patients P2), 3 days before in patient P1 and 4 days before in patient P3 (Table S1).

Histologically, liver architecture was preserved in all patients without any significant portal fibrosis or inflammation. However, an increased number of lobular inflammatory foci was observed in all cases ( $\geq 2$  foci x200; Figure 4A). Immunophenotypical analysis demonstrated an increased number of intrasinusoidal MPO, CD68-positive macrophages and CD206-positive M2 macrophages in the inflammatory foci suggestive of inflammatory leucocytes infiltration (Figure 5A-C).

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3 In all explants, we observed a four-fold mean increased level of *HO1* mRNA compared to  
4 mean levels from 10 control livers (Figure 6A). At the protein level, HO1 is significantly  
5 over-expressed in the explanted livers of 4 patients out of 5. Although patients P1 and P3 did  
6 not receive hemin during the 3 to 4 days preceding surgery, HO1 was maintained at a high  
7 level.  
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15 In all explants, *ALAS1* mRNA levels were in the lower range of the control values (Figure  
16 6A). In contrast, Western blot analyses revealed a significant over-expression of ALAS1 in  
17 the liver of all AIP patients compared to controls ( $P=0.035$ , Figure 6A). P4 and P5 patients  
18 showed the highest induction of ALAS1.  
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26 Perls' blue staining was performed on samples from 3 patients and showed important iron  
27 deposits in hepatocytes and in Kupffer cells (Figure 4B). Moreover, the iron status of 27  
28 recurrent patients with frequent administration of heme arginate was collected. Median  
29 ferritin was 696  $\mu\text{g/L}$  (range: 21-2277) (Table 2), which was above the reference value in  
30 85% of the patients. Twelve patients underwent liver magnetic resonance imaging; of these,  
31 11 of them had confirmed iron overload, and the iron liver content was above 230  $\mu\text{mol/g}$   
32 ( $N<30$ ) in 5 patients. Twelve patients are currently being treated with serial phlebotomies or  
33 chelator administration.  
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## DISCUSSION

Today, the main issue in the medical care of AIP patients is the debilitating condition of patients who present with recurrent attacks. In Europe, 3 to 5 % of individuals with AIP experienced repeated acute attacks [2]. This follow-up studies showed, for the first time, that this percentage reached 7.6% in France. This situation was rare before the introduction of human hemin, and in France, we observed a 4.4-fold increase in the number of recurrent attacks over 30 years beginning with the introduction of heme arginate to the pharmacopeia. All these patients received repeated heme arginate infusion either to treat recurrent acute crises or as a preventive treatment for recurrent attacks.

An acute porphyria attack is considered to be the consequence of an increase in hepatic ALAS1 activity that results in the accumulation of the putatively neurotoxic heme precursors ALA and PBG [3]. Hepatic ALAS1 expression is controlled via negative-feedback regulation by the intracellular free heme pool. This regulation occurs at the transcriptional level [26, 27] and mainly by the modulation of the mitochondrial translocation of ALAS1 [28]. HO1, the key enzyme in heme degradation is one of the major determinants of the level of intracellular-free heme. Heme induces its own catabolism by inhibiting the transcriptional repressor BACH1, allowing the transcription of *HO1* [29].

To date, no study has evaluated the occurrence of chronicity in AIP and experimentally explored the consequences of chronic exogenous heme therapy on the liver. We elucidate the impact of chronic heme administration on the liver in a model of *Hmbs*<sup>-/-</sup> mice. We then extended the investigations to 5 human explanted livers from recurrent AIP patients.

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3 In *Hmbs*<sup>-/-</sup> mice treated repeatedly with heme arginate, HO1 was strongly induced in the liver  
4 both at the mRNA (11-fold) and protein levels (15-fold). This induction exacerbated heme  
5 catabolism and secondarily increased *Alas1* expression. In the 5 explanted livers of AIP  
6 patients, *HO1* mRNA expression was also increased (4-fold). These patients had received  
7 large amount of therapeutic hemin prior to OLT and have induced HO1 response through the  
8 BACH1/NRF2 pathway. At the protein level, HO1 is over-expressed in all patients except  
9 one. Heme arginate half life is assumed to be 10-11h [30] and two patients, P1 and P3  
10 received their last infusion more than five times the half life. Figure 6A shows that although  
11 heme arginate was totally cleared from blood, the two patients exhibited a high level of HO1  
12 induction that could not be due to the last heme arginate infusion exclusively and may  
13 represent a chronic adaptation. Also consistent with the results of the mouse study, increased  
14 expression of ALAS1 was observed chronically in all explanted livers of AIP patients, at the  
15 protein level without mRNA induction which is in favor of a post-transcriptional regulatory  
16 mechanism.  
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35 Liver extracts of heme-treated mice exhibited high levels of pro-inflammatory cytokines  
36 *Tnfa*, *Il-6*, *Il-1β*, and *Il-12p40*. This pattern is a hallmark of MyD88/TLR4 activation by  
37 heme [31]. Likewise, we observed a high expression level of *S100A*, *S100B* and *Marco*  
38 proteins. All these results are consistent with an M1 polarization of pro-inflammatory  
39 infiltrative macrophages in the liver. Evidence of a pro-inflammatory role for heme *in vivo*  
40 was also observed by Wagener *et al.* after a single dose hemin infusion into the tail veins of  
41 BALB/c wild-type mice, leading to the infiltration of leukocytes in the liver [32]. Unlike our  
42 protocol, this study utilized a single high dose of hemin, whereas in the present study, we  
43 investigated the effects of a cumulative dose of hemin over a 2-month period and observed  
44 chronic and complex inflammatory lesions.  
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5 The liver extracts from heme-treated mice also showed an increased expression of Mrc1,  
6 Mgl1 and the Il-10 anti-inflammatory cytokines, which were suggestive of M2 polarization of  
7 macrophages. Human-explanted livers from AIP patients also highly expressed MRC1  
8 (CD206), which was compatible with a M2 polarization. One distinguishing characteristic  
9 between M2 and M1 macrophage polarization is the intracellular redox status [33]. The  
10 oxidation status observed in liver extracts of heme-treated mice is characteristic of an M2  
11 polarization (a reduced GSH/GSSG ratio, a high level of 8-isoprostane and an induction of  
12 *Cybb* and *Rac2*; Figure 3B and Table S2). Altogether, these data support the idea of a  
13 complex inflammatory pattern consisting of an M1/Th1 pro-inflammatory pattern associated  
14 with a predominant M2/Th2 anti-inflammatory response, according to the oxidation status.  
15 Several studies showed that the anti-inflammatory cytokine Il-10 induces HO1 [34-36] which  
16 could amplify the effects of heme *per se* on HO1 expression.  
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33 Also notable was the observation of the presence of iron deposition in livers of both heme-  
34 treated mice and AIP patients exposed to chronic heme infusions (Figures S1 and 4B).  
35 Repeated hemin infusion is responsible for a high cumulative dose of iron (Table 2). Marsden  
36 *et al.* have shown that there was a significant correlation between the serum ferritin  
37 concentration and number of doses that were administered [15]. In the explanted livers, iron  
38 overload was observed both in hepatocytes and in K upffer cells where it presented as large  
39 aggregates. In fact, Vinchi *et al.* showed that during intravascular hemolysis leading to free  
40 heme excess, the liver is the most susceptible organ to heme overload, and when hemopexin  
41 capacity is exceeded, heme accumulates mainly in K upffer cells [37]. In line with our results,  
42 three well-documented studies concerning recurrent AIP patients reported also that regular  
43 hemin infusions trigger variable liver iron overload [8, 9, 38].  
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3 **Different studies have shown that iron overload impairs the hepatic mitochondrial**  
4 **metabolism [39, 40]. High amount of free iron released by HO-1 catalyzes the formation**  
5 **of reactive oxygen species and is responsible for alteration of mitochondrial respiration**  
6 **in rodent submitted to systemic inflammation [41]. Thus, liver iron overload, as**  
7 **observed in recurrent AIP patient, could worsen the impairment of the respiratory**  
8 **chain and the tricarboxylic acid cycle of the liver mitochondrias during an acute attack,**  
9 **as previously shown in the AIP mouse model [42].**  
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18 Oxidative stress, as observed in Figure 3B, induces the dissociation of the stress sensitive  
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20 KEAP1 protein from NRF2. In turn, NRF2 translocates to the nucleus and then transactivates  
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22 *HO1* expression through the AREs sequences and consequently enhances endogenous free  
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24 heme degradation [43].  
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28 ~~HO1 heme catabolism produces antioxidant bilirubin, carbon monoxide and apoferritin but~~  
29 ~~also ferrous redox active iron, catalyzing the formation of reactive oxygen species.~~  
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## 35 **CONCLUSION**

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37 AIP is considered as an intermittent disease, but 7.6% of patients have a chronic life-  
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39 threatening debilitating condition. Our study highlights a key role of hemin infusions in the  
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41 pathophysiology of AIP chronic attacks. Nevertheless, hemin is the most effective treatment  
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43 for acute neurovisceral attacks and is life-saving. The introduction of heme arginate decreased  
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45 mortality at a price of increasing recurrent attacks. Hemin treatment needs therefore to be  
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47 restricted to patients with severe forms of AIP crises with the occurrence of progressive signs  
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49 of a central or peripheral neurological alteration, profound hyponatremia or hyperalgesic  
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51 acute crisis unresponsive to symptomatic treatment. Altogether, we bring convergent  
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53 arguments showing that chronic exogenous heme infusions were responsible for a  
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3 physiological response which activates chronically HO1 expression. The 3 factors that could  
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5 be implicated in HO1 induction are heme *per se*, the inflammation characterized by an  
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7 adaptive M2 macrophage polarization promoting the IL-10-HO1 amplifying loop and the  
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9 oxidative stress generated by iron overload (Figure 6B). In the context of AIP, exacerbated  
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11 free heme degradation would reduce the free heme pool, which in turn would up-regulate  
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13 ALAS1, promote ALA and PBG overproduction and finally, exacerbate porphyric symptoms.  
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15 In humans, the HO1 promoter contains a (GT)(n) dinucleotide repeat that is related to HO1  
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17 expression and activity, and it would be tempting to speculate that this polymorphism could  
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19 modulate the basal level of the free heme pool and in turn the penetrance and the severity of  
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21 AIP. The present data that demonstrates the role of chronic inflammation on HO1 activation  
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23 after repeated heme therapy is crucial for the improvement of recurrent AIP patient care.  
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25 Subsequent development of new and safe ALAS1 and/or HO1 inhibitors would greatly  
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27 improve AIP treatment.  
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3 **CONFLICT OF INTEREST STATEMENT**

4 **LG, JCD, PH, ES and AKA were funded for attending meeting related to ongoing**  
5 **clinical trial by Alynlam Pharmaceuticals. Other authors have no conflicts of interest to**  
6 **declare.**  
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13 **ACKNOWLEDGEMENTS**

14  
15 We are very grateful to Professor Urs Meyer and Doctor Anne-Kathrin Peyer for having  
16 provided the living *Hmbs*<sup>-/-</sup> mice, to Eric Couchi for his excellent supervision of animal  
17 breeding and treatments and to Sylvie Simonin for urinary ALA and PBG measurements.  
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## FIGURE LEGENDS

### Figure 1 - Increasing numbers of AIP patients with recurrent acute attacks.

The proportion of recurrent AIP patients has increased in the past 40 years in the French Center of Porphyrrias. Recurrent AIP patients already existed before the introduction of human hemin but at lower numbers.

### Figure 2 - Repeated human hemin injection induces liver HO1 expression in the AIP mouse model.

A. Each group consists of 6 C57BL/6 Hmbs-deficient female mice. Arrows represent the intraperitoneal injection of either saline, phenobarbital (PB) 100 mg/kg, heme arginate (HA) 8 mg/kg or excipients (arginine, ethanol, propylene glycol and water). All groups of animals were sacrificed at day 5 on the eighth week, except for 3 mice in the “PB/Saline” group which were sacrificed at day 3 on the eighth week, just after the last phenobarbital injection (“PB/Saline D3” group).

B. Every day for 4 different weeks, porphobilinogen was measured in pooled fresh urine samples. Massive excretion of porphobilinogen in urine 24 hours after each phenobarbital injection (arrows) in Hmbs-deficient mice was observed. Hmbs-deficient mice treated only by saline (black square line) showed very low excretion of porphobilinogen. The color of each graph corresponds to the color of the mouse group.

C. Western blot and densitometry analysis of Alas1 from liver lysates of Hmbs-deficient mice. An over-expression of Alas1 was observed in the liver samples from mice sacrificed just after the last phenobarbital induction (“PB/Saline D3” group). Two days after the phenobarbital injection (“PB/Saline” group), Alas1 expression returned to a basal level. However, after repeated heme treatment, Alas1 expression in the liver was significantly higher (“PB/HA” group).

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3 D. Liver *HO1* mRNA quantification via RT-qPCR. *HO1* mRNA was increased (11-fold) in  
4 the liver samples of *Hmbs*<sup>-/-</sup> mice induced with phenobarbital and treated by heme arginate.

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7 E. Western blot and densitometry analysis confirm that HO1 is over-expressed in the liver of  
8  
9 *Hmbs*<sup>-/-</sup> mice treated with hemin compared to excipients or saline.

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11 Data information: Statistically significant difference \*  $P < 0.05$  or \*\*  $P < 0.01$ .

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16 **Figure 3 - Inflammation and oxidative stress induction in the liver samples of AIP mouse**  
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18 **model treated chronically with human hemin.**

19  
20 A. Myeloperoxidase and leukotriene LTE4 quantification in the liver of the mice.  
21  
22 Myeloperoxidase and leukotriene were highly increased in the heme arginate group.

23  
24 B. Markers of oxidative stress in the liver of the mice. In the heme arginate group, the  
25  
26 reduced-oxidized glutathione ratio was significantly decreased, and 8-isoprostane was  
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28 increased.

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31 Data information: Statistically significant difference \*\*  $P < 0.01$ .

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35 **Figure 4 - Liver iron overload in AIP patients receiving frequent human hemin**  
36  
37 **administration.**

38  
39 A. HES staining (x200) showing normal liver architecture with a non-fibrous portal tract (PT)  
40  
41 and the presence of intralobular inflammatory cells (arrows).

42  
43 B. Perls' staining (x200) showing macrophage and hepatocyte iron accumulation.

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48 **Figure 5 - Macrophage activation in the liver samples of AIP patients receiving frequent**  
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50 **human hemin administration.**

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52 A. Increased infiltration of MPO-positive inflammatory cells inside the sinusoids compared to  
53  
54 the few isolated positive cells present in the control sample (x200).

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3 B. Increased infiltration of CD68-positive macrophages inside the sinusoids compared to the  
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5 few isolated positive cells present in control sample (x200).

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7 C. Intense positive signal in the endothelial sinusoidal cells throughout the lobule. In addition,  
8  
9 intrasinusoidal inflammatory cells are CD206-positive (arrows) in samples from AIP patients  
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11 compared to control samples (x400).  
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16 **Figure 6 - HO1 and ALAS1 activation in the liver samples of recurrent AIP patients.**

17  
18 A. *HO1* mRNA is highly increased in the liver of recurrent AIP patients who had a liver  
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20 transplantation (n=5) when compared to controls (n=10, healthy tissue around hepatic  
21  
22 adenoma from non-porphyric patients). At the protein level, HO1 was induced in the liver of  
23  
24 4 patients out of 5. ALAS1 protein is significantly over-expressed in all the explanted liver of  
25  
26 recurrent AIP patients (n=5) when compared to controls (n=10). *ALAS1* mRNA exhibited no  
27  
28 difference between AIP patients and control, which underlines the predominant regulation of  
29  
30 ALAS1 at the post-transcriptional level. All AIP patients had been treated with human hemin  
31  
32 between 3 to 96 h before transplantation.  
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35  
36 B. Schema of the hypothetical mechanism of HO1 induction in the liver of AIP patients after  
37  
38 cumulative doses of hemin. The 3 factors that could be implicated in HO1 induction are heme  
39  
40 *per se*, the inflammation characterized by an adaptive M2 macrophage polarization promoting  
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42 the IL-10-HO1 amplifying loop and the oxidative stress generated by iron overload.  
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## TABLES

**Table 1. Expression of cytokines and macrophage differentiation markers in the livers of hemin treated mice (PB/HA group) compared to mice treated with saline (PB/Saline group). NS: not significant ( $P > 0.1$ ).**

	Gene	mRNA level	Protein level	Fold change	<i>P</i>
<b>Pro-inflammatory markers / M1 macrophage polarization</b>	Il-1	x		1.3	NS
	Inf $\gamma$	x		0.8	NS
	Il-4		x	1.9	NS
	<b>Il-1<math>\beta</math></b>		x	<b>1.3</b>	<b>0.0260</b>
	<b>Il-6</b>	x		<b>3.3</b>	<b>0.0022</b>
			x	<b>1.7</b>	<b>0.0411</b>
	<b>Il-12p40</b>		x	<b>2.6</b>	<b>0.0260</b>
<b>Anti-inflammatory markers / M2 macrophage polarization</b>	<b>Tnf<math>\alpha</math></b>	x		<b>3.0</b>	<b>0.0087</b>
			x	<b>1.3</b>	<b>0.0600</b>
	Arg1	x		1.0	NS
	<b>Il-10</b>	x		<b>3.3</b>	<b>0.0022</b>
			x	<b>1.7</b>	<b>0.0900</b>
	<b>Il-13</b>		x	<b>4.1</b>	<b>0.0411</b>
	<b>Mrc1</b>	x		<b>1.5</b>	<b>0.0022</b>
	<b>Mgl1</b>	x		<b>1.8</b>	<b>0.0022</b>
	<b>Atf1</b>	x		<b>1.2</b>	<b>0.0159</b>
<b>Cd91</b>	x		<b>1.2</b>	<b>0.0550</b>	
<b>Cd163</b>	x		<b>1.6</b>	<b>0.0317</b>	
<b>HO1</b>	x		<b>11.7</b>	<b>0.0022</b>	
			x	<b>6.5</b>	<b>0.0238</b>

**Table 2. Iron status of 27 AIP patients with frequent administration of heme arginate.**

Dosage schedule of heme arginate. M: monthly, MX2: twice per month, MX3: three times per month, MX4: four times per month, MX8: eight times per month. NP: not performed.

Sexe	Age	Age of diagnosis	Heme arginate treatment (250mg)	Ferritin µg/l F: 3-105 M: 5-250	Liver MRI Iron liver content µmol/g (N<30)	Serial phobotomies	Chelator treatment
F	29	26	MX2 - MX4	2277	230: <b>high overload</b>	<b>+</b>	
F	62	53	M - MX2	1962	260: <b>high overload</b>	<b>+</b>	
F	50	30	MX3	1360	<b>moderate overload</b>	no (due to low Hb)	
F	56	34	MX3	1329	290: <b>high overload</b>	<b>+</b>	
F	42	30	MX4	1203	240: <b>high overload</b>	<b>+</b>	
F	37	21	MX8	1200	NP		
M	49	34	MX4	1003	NP	<b>+</b>	
F	50	40	MX4	986	NP		<b>+</b>
M	48	32	MX4	975	90: <b>mild overload</b>	<b>+</b>	
F	43	22	MX2	900	270: <b>high overload</b>	<b>+</b>	
F	66	60	when crisis	886	NP	<b>+</b>	
F	41	33	MX8	700	NP		
F	39	20	MX4	630	NP	<b>+</b>	
F	51	29	M – MX3	621	90: <b>mild overload</b>		
F	43	27	MX3	542	<b>mild overload</b>	<b>+</b>	
F	38	34	MX3	436	100: <b>moderate overload</b>		
M	54	37	M	384	130: <b>moderate overload</b>	<b>+</b>	
F	33	18	MX4	316	NP		
F	45	34	when crisis	253	NP		
F	49	38	MX2	198	NP		
F	59	22	when crisis	190	NP		
F	43	21	MX2 – MX3	161	25: No overload		
F	33	30	when crisis	112	NP		
F	36	29	when crisis	73	NP		
F	27	22	MX2	44	NP		
F	34	26	when crisis	40	NP		
F	34	23	when crisis	21	NP		

**Mean****696**

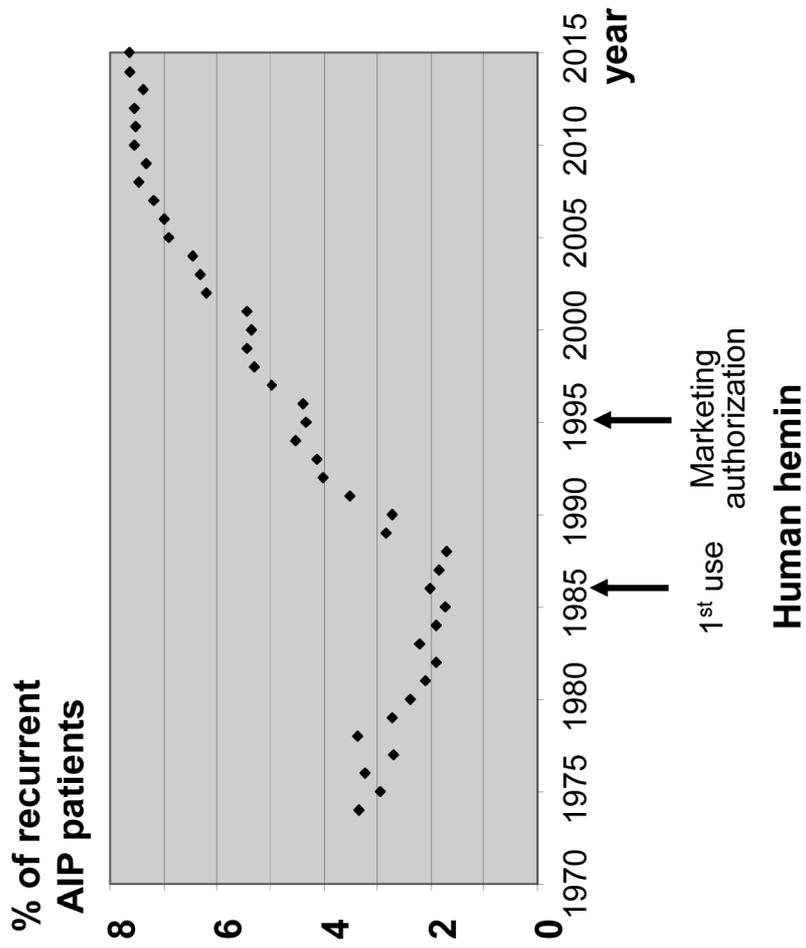
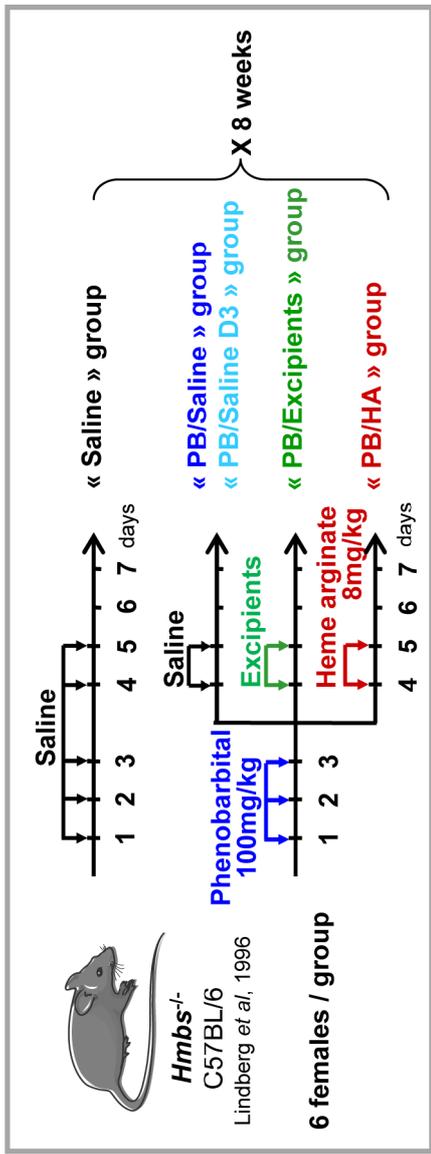


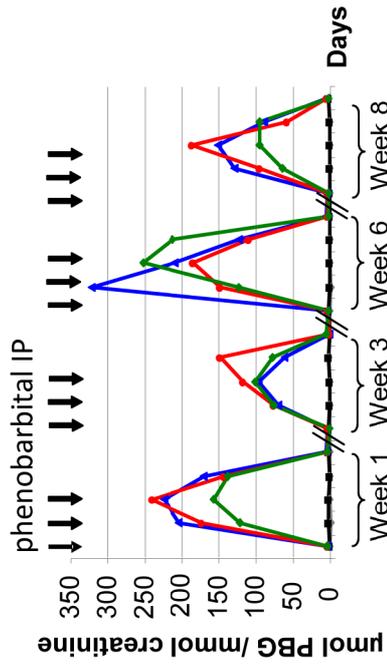
Figure 1

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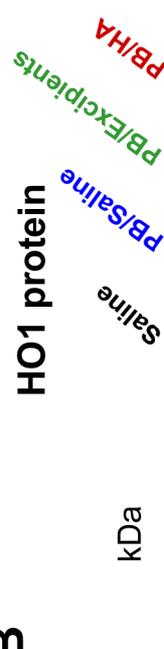


**A**

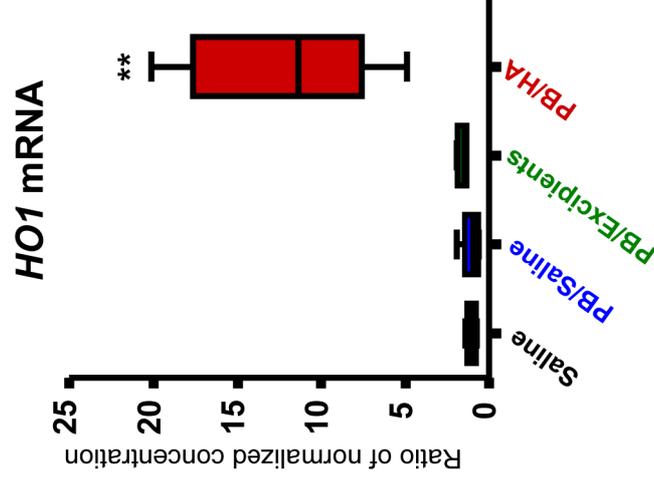
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**Alas1 protein**



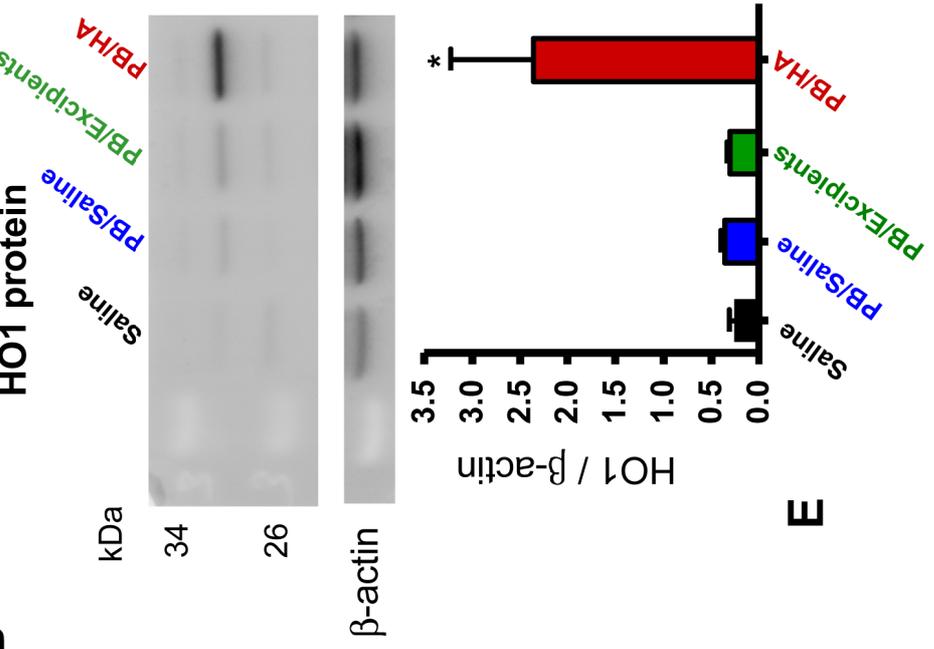
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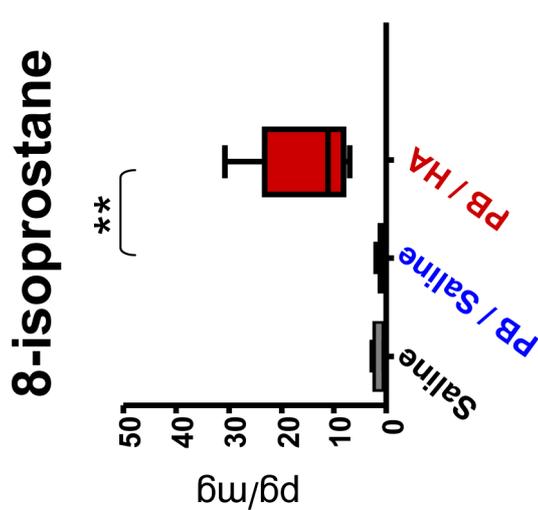
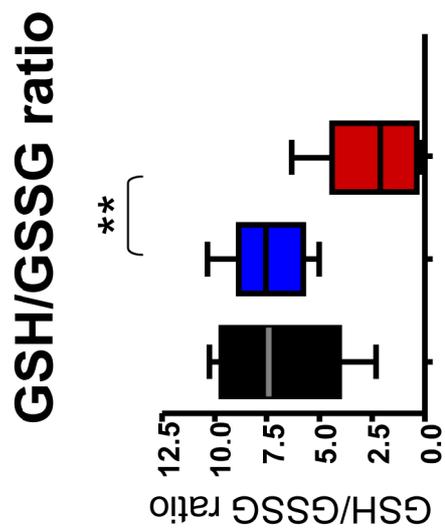
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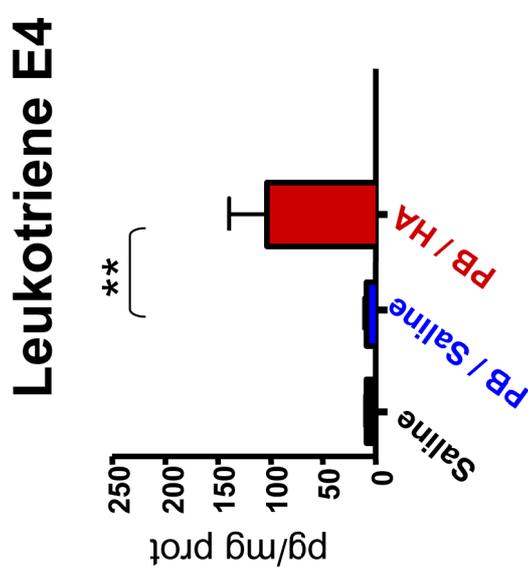
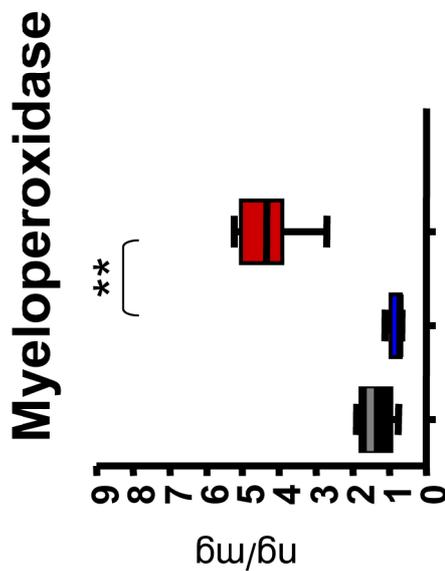
**E**



**Figure 2**



**B**



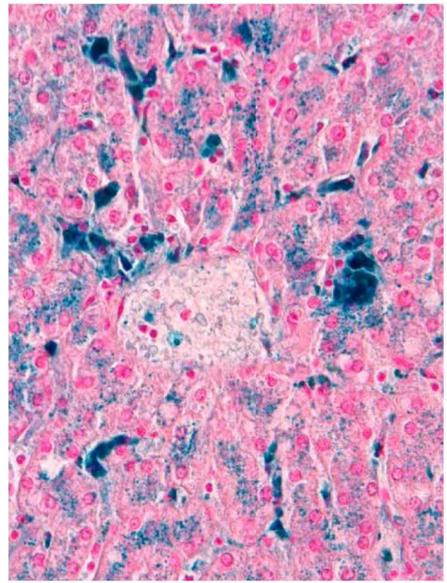
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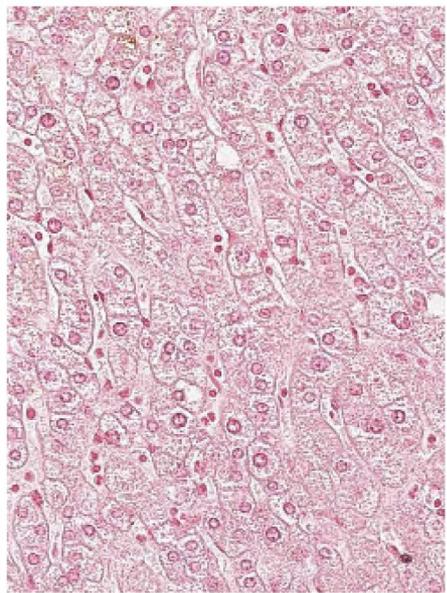
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**B**

**Perls staining**



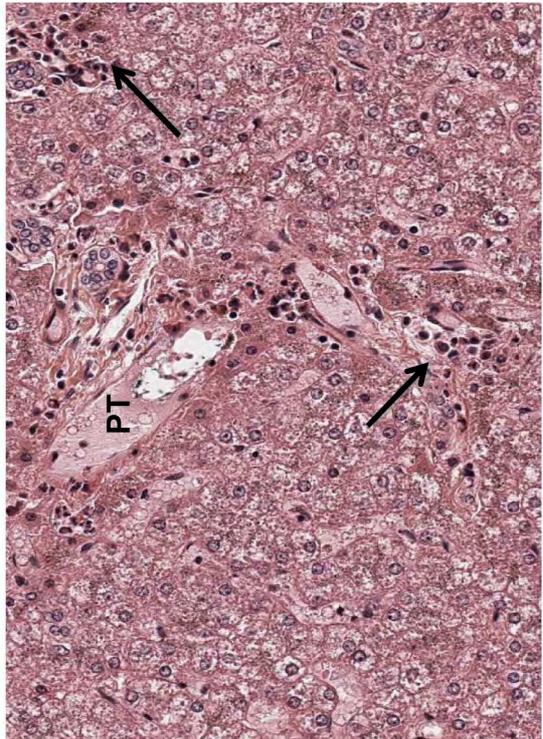
AIP patient



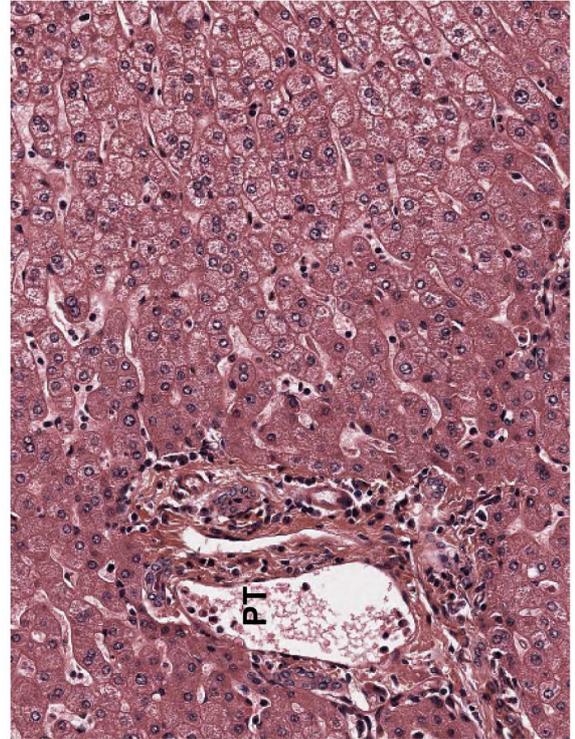
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**HES staining**



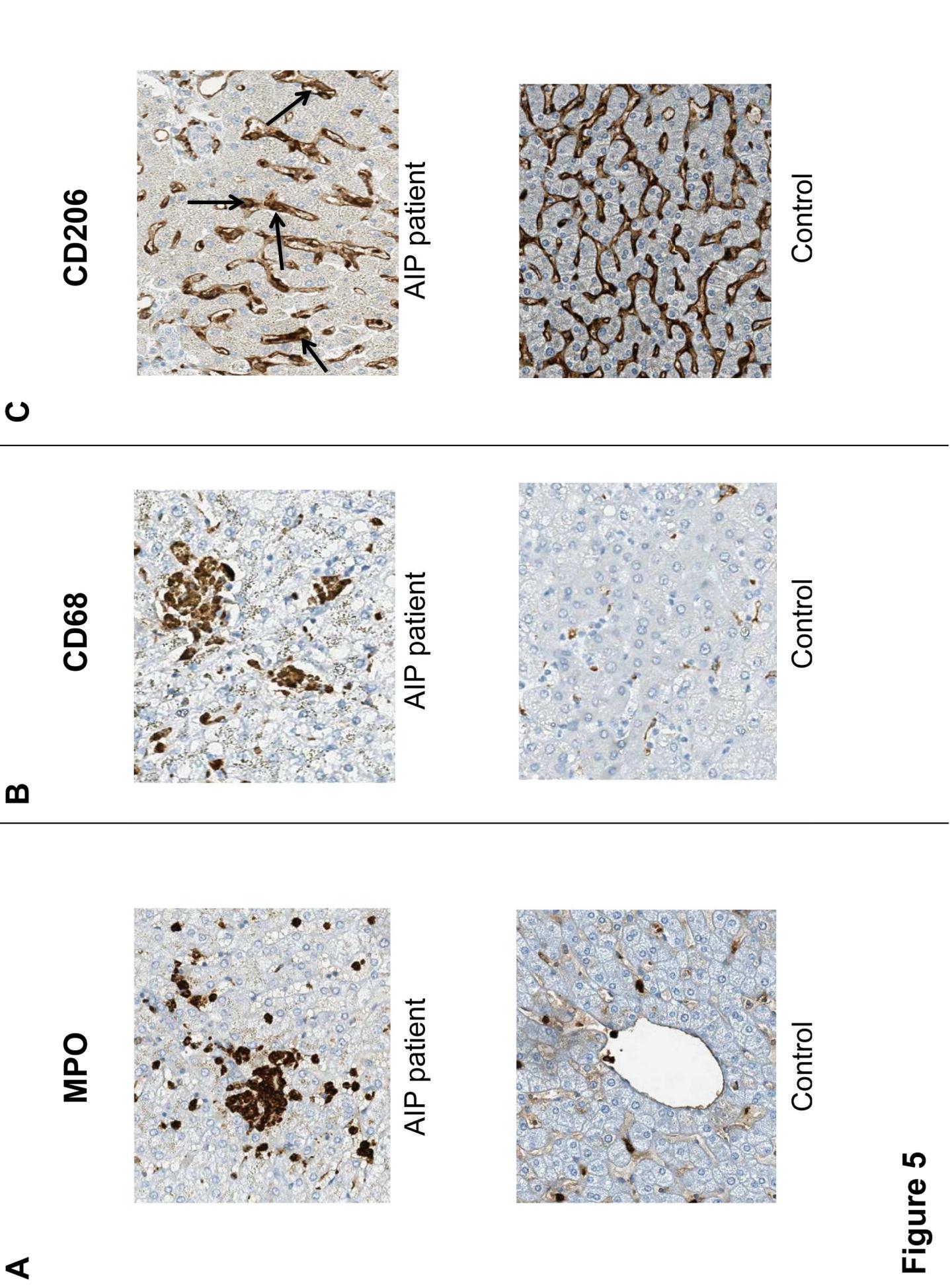
AIP patient



Control

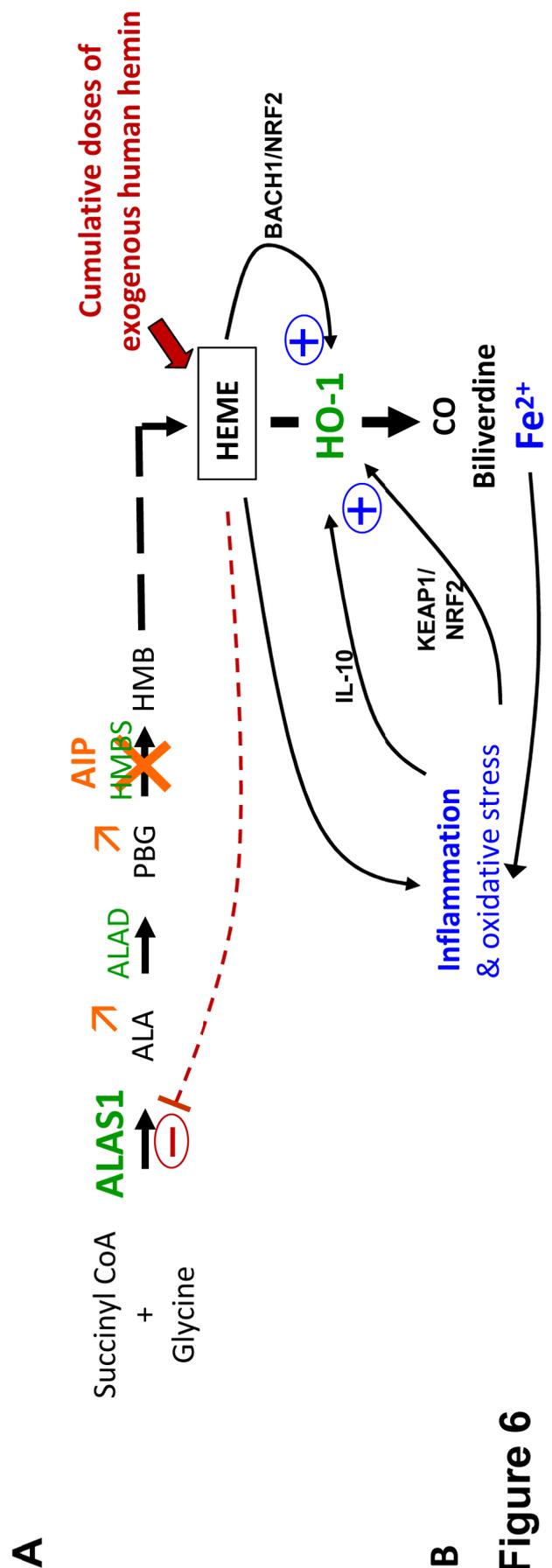
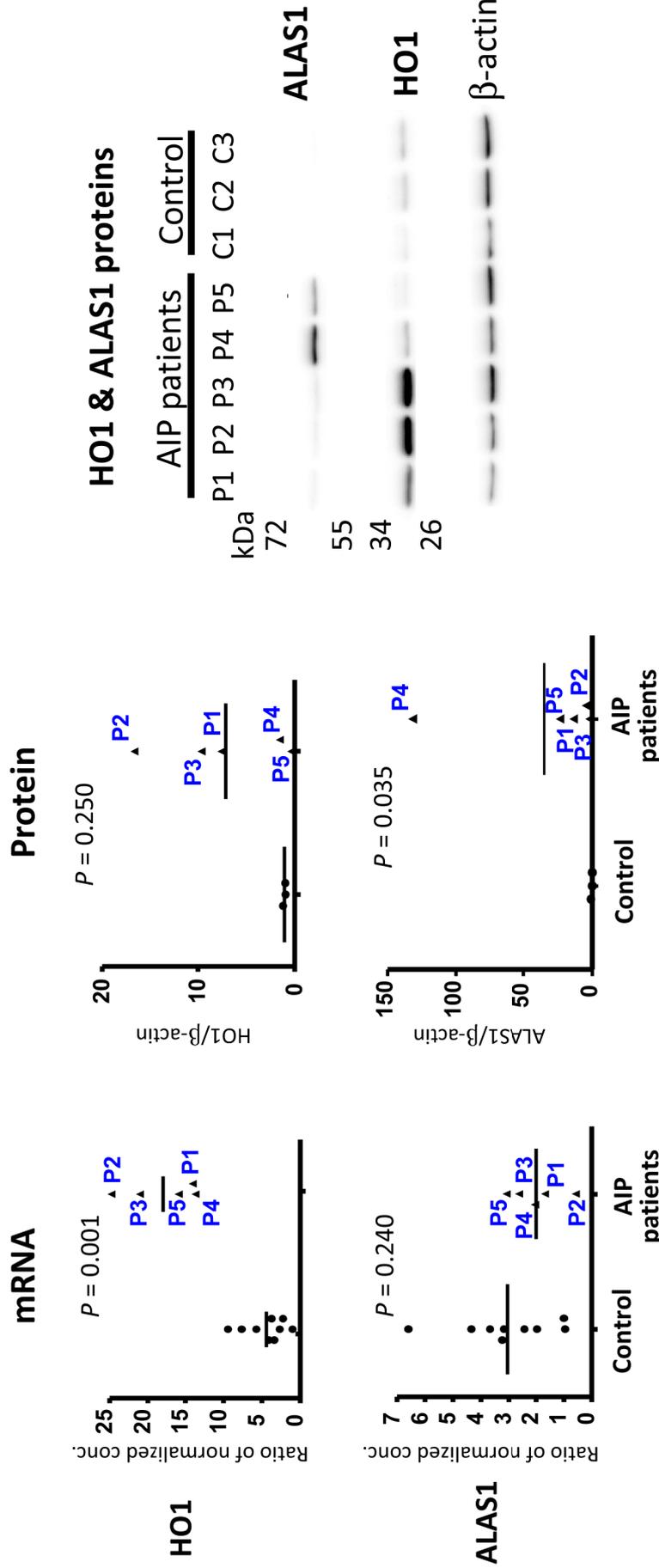
**Figure 4**

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**Figure 5**

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**Figure 6**

## Supplementary tables

**Table S1. Heme arginate infusion in recurrent AIP patients who underwent a liver transplantation.**

<b>Patient</b>	<b>Origin</b>	<b>HMBS mutation</b>	<b>Heme arginate infusion frequency</b>	<b>Time between last heme arginate infusion and OLT</b>
<b>P1</b>	France	c.518G>A p.(Arg173Gln)	250 mg x 2 / week	72h
<b>P2</b>	France	c.291delG p.(Lys98Argfs*37)	250 mg x 4 / 3 weeks	24h
<b>P3</b>	Norway	c.593G>A p.(Trp198*)	175 mg x 4 / 4 weeks	96h
<b>P4</b>	Sweden	c.104_105insGTCT p.(Asp36Serfs*18)	138 mg x 1 / week	3h
<b>P5</b>	France	c.687_689delinsT p.(Gln229Hisfs*20)	250 mg x 1 / week	3h

**Table S2. Hepatic genome-wide transcriptomic analysis after chronic heme arginate infusion in *Hmbs*<sup>-/-</sup> mice.** Down-regulated and up-regulated hepatic transcripts of mice treated with heme arginate (PB/HA group) compared to mice treated with saline (PB/Saline group).

Down-regulated transcripts			Up-regulated transcripts		
Gene Symbol	Fold change	P-value	Gene Symbol	Fold change	P-value
A030001D16Rik	0,01	0,083	3110009E18Rik	29,86	0,077
Cyp2c55	0,04	0,013	4932442A14Rik	29,86	0,039
Cyp2c65 Cyp2c66	0,05	0,063	Dip2	25,99	0,030
Osbp1a	0,05	0,091	Cyp4a12	10,56	0,094
Pitx3	0,07	0,067	Hmox1 = HO1	9,19	0,042
2310016D03Rik	0,08	0,055	Tmco3	6,96	0,077
9130211I03Rik	0,08	0,068	Akr1b8	6,96	0,001
4931420D14Rik	0,11	0,081	Fbxl21	5,66	0,092
4932425I24Rik	0,13	0,082	H2-DMa	4,92	0,076
B230334C09Rik	0,13	0,094	Clec4n	4,59	0,068
1700067K01Rik	0,14	0,0001	S100a9	4,59	0,084
Cyp2c29	0,14	0,005	Marco	4,29	0,022
Akr1b7	0,15	0,088	Bmp6	4,00	0,068
LOC632862	0,20	0,094	Cybb	3,48	0,093
Cdkn3	0,22	0,052	Trim13	3,03	0,057
Neu2	0,22	0,094	2310021P13Rik	3,03	0,082
V1rd3	0,23	0,061	Spic	3,03	0,095
Nr4a1	0,23	0,092	6330405D24Rik LOC625403	3,03	0,040
Fbxw2	0,23	0,071	LOC270040 LOC619780	2,83	0,046
Col1a1	0,29	0,068	S100a8	2,83	0,082
Tcf15	0,29	0,083	Rac2	2,83	0,092
Mapk13	0,29	0,071	LOC628298 LOC637129	2,83	0,020
Ces2	0,29	0,069	Hcls1	2,64	0,014
Cyp2c54	0,31	0,031	LOC622384 LOC634810	2,64	0,020
Cyp3a16	0,31	0,048	Sox18	2,64	0,098
LOC639023 Cyp2c50 Cyp2c37 Cyp2c54	0,31	0,049	Itgb2	2,46	0,022
Slc23a3	0,33	0,058	Cyp4b1	2,46	0,070
4931406C07Rik	0,33	0,022	Cd44	2,46	0,069
Wfikn2	0,33	0,052	Gngt2	2,46	0,057
Cyp3a44	0,35	0,046	2310043N10Rik	2,46	0,013
Ces2 LOC628079 LOC637641	0,35	0,036	Vsig4	2,46	0,040
Cyp3a11 Cyp3a44 Cyp3a41	0,35	0,072	Cend1	2,30	0,057
Birc5	0,38	0,096	Hps3	2,30	0,092
Cyp2c37	0,38	0,065	Ppap2a	2,30	0,079
Cyp1a2	0,38	0,057	Stab1	2,30	0,069
Acot1	0,44	0,094	2010110P09Rik	2,30	0,051
Gstm3	0,44	0,099	Id1	2,30	0,057

Gstm4	0,47	0,081	Arrb2	2,14	0,095
Ces6	0,47	0,094	Ckap4	2,14	0,084
Gstp1	0,47	0,071	Clec12a	2,14	0,052
Abcc3	0,47	0,084	Eltf1	2,00	0,095
Sult5a1 LOC639904	0,50	0,088	Fcna	2,00	0,090
B230333C21Rik	0,50	0,096	Srebf1	2,00	0,077
<b>Total : 43 genes</b>			Folr2	2,00	0,096
			Msn	2,00	0,087
			Aacs	1,87	0,082
			Lgals1	1,87	0,080
			Cryl1	1,87	0,094
			Id2	1,74	0,070
			Stim2	1,74	0,065

**Total : 50 genes**

For Peer Review

**Table S3. Classification of the transcripts according to biological process.** Up-regulated (in red) and down-regulated (in green) hepatic transcripts of mice treated with heme arginate (PB/HA group) compared to mice treated with saline (PB/Saline group).

Analysis performed by g:Profiler software. FDR: False Discovery Rate. In bold are biological pathways linked to inflammation.

FDR <i>P</i> -value	term ID	Pathway genes (%)	Significantly overrepresented biological process	Genes
2,31E-04	GO:0070488	75,00	Neutrophil aggregation	<b>S100A8,S100A9,GSTP1</b>
1,25E-02	GO:0002523	25,00	Leukocyte migration involved in inflammatory response	<b>ITGB2,S100A8,S100A9</b>
4,74E-03	GO:0019373	12,90	Epoxygenase P450 pathway	<b>CYP2C29,CYP2C55,CYP2C37,CYP2C54</b>
2,40E-05	GO:0042738	11,30	Exogenous drug catabolic process	<b>CYP2C29,CYP2C55,CYP4B1,CYP1A2,CYP2C37,CYP2C54</b>
1,65E-02	GO:0043392	9,50	Negative regulation of DNA binding	<b>HMOX1(HO1),ID2,ID1,WFIKKN2</b>
3,95E-09	GO:0070887	1,30	Cellular response to chemical stimulus	<b>ITGB2,COL1A1,CYP2C29,GSTM3,CD44,HMOX1(HO1),BIRC5,SREBF1,ID2,HCLS1,NR4A1,CYP2C55,GSTM4,AACS,2010110P09RIK,MSN,CYP1A2,RAC2,BMP6,CYP2C37,ID1,WFIKKN2,S100A8,S100A9,ARRB2,CYP2C54,CCND1,GSTP1</b>
1,62E-03	GO:0002376	1,00	Immune system process	<b>ITGB2,CD44,HMOX1(HO1),CYBB,ID2,HCLS1,CLEC4N,MARCO,FCNA,MSN,RAC2,TRIM13,H2-DMA,BMP6,VSIG4,CLEC12A,S100A8,S100A9,ARRB2,LGALS1</b>

3,79E-03	GO:0048522	0,70	Positive regulation of cellular process	ITGB2, COL1A1, SPIC, CD44, HMOX1(HO1), BIRC5, SREBF1, ID2, HCLS1, NR4A1, CLEC4N, PITX3, AACCS, 2010110P09RIK, MSN, FOLR2, RAC2, TRIM13, H2-DMA, BMP6, STIM2, ID1, SOX18, S100A8, S100A9, ARRB2, LGALS1, CCND1, NEU2, GSTP1
1,94E-04	GO:0044699	0,40	Single-organism process	ITGB2, COL1A1, CYP2C29, SPIC, MAPK13, CD44, HMOX1(HO1), CYBB, BIRC5, SREBF1, ID2, ABCC3, PPAP2A, CRYL1, HCLS1, NR4A1, CLEC4N, CYP2C55, PITX3, SLC23A3, MARCO, FCNA, HPS3, CYP4B1, AACCS, AKR1B8, 2010110P09RIK, MSN, CYP1A2, FOLR2, RAC2, TRIM13, CDKN3, H2-DMA, TMCO3, CYP3A16, GNGT2, BMP6, STIM2, ELTD1, CYP2C37, STAB1, ID1, WFIKKN2, VSIG4, OSBPL1, SOX18, AKR1B7, CYP3A44, S100A8, S100A9, ARRB2, CYP2C54, TCF15, LGALS1, CCND1, ACOT1, NEU2, GSTP1

**Table S4. Proteome analysis after chronic heme arginate infusions in *Hmbs*<sup>-/-</sup> mice.**

Differentially expressed plasma proteins in mice treated with heme arginate (PB/HA group) compared to the PB/Saline group.

<b>Protein</b>	<b>Function</b>	<b>Fold change</b>	<b><i>P</i></b>
<b>Haptoglobin precursor (Hap)</b>	Positive acute-phase protein of inflammation	4.37	0.005
<b>Serum amyloid P-component precursor (Apcs)</b>	Positive acute-phase protein of inflammation	3.65	0.001
<b>Complement C3 precursor</b>	Positive acute-phase protein of inflammation	3.65	0.001
<b>Apolipoprotein A1 precursor</b>	Major protein component of high density lipoprotein (HDL)	3.3	0.003
<b>Proteasome subunit alpha type-1 (Psa1)</b>	Proteasome	2	0.008
<b>Alpha-1-microglobulin/bikunin precursor (Ambp)</b>	Processed into: alpha-1-microglobulin (may play a role in the regulation of inflammation) and bikunin (urinary trypsin inhibitor)	1.9	0.034
<b>Alpha- feto protein (Afp)</b>	Fetal glycoprotein Tumor marker	0.77	0.0007
<b>Prealbumin precursor = Transthyretin (Ttr)</b>	Negative acute-phase protein of inflammation	0.74	0.005
<b>Alpha-1-antitrypsin 1-1 precursor (A1at1)</b>	Positive acute-phase protein of inflammation	0.43	0.005
<b>Coagulation factor X precursor (Fa10)</b>	Blood coagulation	0.43	0.005
<b>Complement factor I precursor (Cfa1)</b>	Inactivates complement subcomponents C3b, iC3b and C4b	0.43	0.005

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3 **Supplementary figure legend**  
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7 **Figure S1 - Iron overload in the liver samples of AIP mouse model treated chronically**  
8 **with human hemin.**  
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11 A. Iron content measurement in the liver of the mice. Hmbs-deficient mice chronically  
12 treated with heme arginate showed a significantly higher iron content in the liver.  
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16 B. Liver *Bmp6*, *Id1* and *Hepcidin (Hepc1)* mRNA quantification by RT-qPCR. In response to  
17 iron overload, *Bmp6* and *Id1* mRNA were significantly increased in the liver of the Hmbs-  
18 deficient mice induced with phenobarbital and treated with heme arginate, and *Hepcidin*  
19 expression tended to be higher in heme arginate group.  
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24 Data information: The results are presented as box plots showing the median, quartiles, the  
25 90th and the 10th percentiles. Statistically significant difference \*  $P < 0.05$ .  
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## Supplementary materials and methods

### Materials

Phenobarbital (GARDENAL<sup>®</sup> Sanofi-Aventis, Paris, France) consists of 40 mg of lyophilized powder reconstituted with 2 mL of water for injection. Heme arginate (NORMOSANG<sup>®</sup> Orphan Europe, Paris-La Défense, France) is an arginine-stabilized form of human hemin containing 250 mg of human hemin and excipients (arginine 267 mg; ethanol 96% 1000 mg; propylene glycol 4000 mg and water for injection) per ampoule.

### Determination of mouse ALA and PBG urine levels

Fresh urine samples were harvested before treatment began and every day during weeks 1, 3, 6 and 8 of treatment. ALA and PBG levels in pooled urine samples were analyzed via sequential ion-exchange chromatography using the ALA/PBG column Test Kit (Bio-Rad, Hercules, CA, USA). Urinary creatinine was measured with a Dimension-RXL analyzer (Siemens Healthcare, Erlangen, Germany).

### Microarray analysis of gene expression in the mouse liver

The mouse liver transcriptome was analyzed using Mouse Genome Survey Arrays v2.0 (Applied Biosystems, Foster City, CA, USA), containing 32,996 probes (60-mer oligonucleotide), which represented a set of 32,381 annotated mouse genes. (i) RNA isolation for microarray experiments: total RNA was isolated from mouse liver using the NucleoSpin RNA L (Macherey-Nagel, Düren, Germany) and concentrated using Rneasy MinElute Cleanup kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Quantity of the isolated total RNA was determined by spectrophotometry (Nanodrop instrument, Thermo Scientific, Waltham, MA, USA) whereas assessment of RNA quality and integrity was

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3 determined with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).  
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5 All RNA samples presented a RIN (RNA Integrity Number) between 7 to 9. (ii) RNA  
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7 labeling, hybridization, and detection: 20 µg of total mouse liver RNA sample were first  
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9 subjected to chemiluminescence RT labeling (Applied Biosystems Chemiluminescent RT  
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11 labeling kit, Foster City, CA, USA). Digoxigenin-labeled cDNAs were then hybridized and  
12  
13 detected according to the supplied protocols (Applied Biosystems Chemiluminescence  
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15 Detection kit, Foster City, CA, USA). A total of 12 independent experiments were performed  
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17 for the two different biological conditions (PB/HA and PB/Saline groups). (iii) Transcriptome  
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19 Data Analysis: Applied Biosystems Expression Array System Software v1.1.1 was used to  
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21 acquire the chemiluminescence and the fluorescence images and to perform primary data  
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23 analysis. In addition, we renormalized the resulting data according to the logarithmic signal  
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25 median, once more, after having removed the probes for which the Applied Biosystems  
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27 Software has set flags equal to or greater than  $2^{12}$ , indicating compromised or failed  
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29 measurements, and signals from control spots.  
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### 35 **Quantitative real-time RT-PCR analysis of mouse and human liver samples**

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37 Total RNA was isolated from human liver samples using the RNeasy Mini Kit (Qiagen,  
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39 Hilden, Germany) and from mouse liver samples using NucleoSpin RNA L (Macherey-Nagel,  
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41 Düren, Germany) according to the manufacturer's protocol. In all, 100 ng of extracted total  
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43 RNA was reverse transcribed using SuperScript™ II Reverse Transcriptase (Invitrogen,  
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45 Carlsbad, CA, USA). Quantitative PCR amplification was conducted using LightCycler® 480  
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47 SYBR Green I Master kit (Roche, Basel, Switzerland) according to the manufacturer's  
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49 instructions, with 0.25 µM primers in a 20 µL final reaction volume. Thermocycling was  
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51 performed in a 96-well format in the LightCycler® 480 Instrument (Roche, Basel,  
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53 Switzerland), initiated by a 5 min incubation at 95°C, followed by 40 cycles (95°C, 10 s;  
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60°C, 20 s; 72°C, 30°C) with a single fluorescent reading taken at the end of each cycle. Each run was completed with a melting curve analysis to confirm the specificity of amplifications. All samples were amplified in duplicates from the same cDNA preparation and the mean value was used for further analysis. Crossing points and concentrations for each transcript were determined using the 2<sup>nd</sup> derivative maximum analysis on the LightCycler 480 software. For each transcript standard curves were determined with 4 serial dilutions of a reference cDNA. Amplification efficiencies were then calculated for each gene and each primer pair was 80 to 100% efficient. Most stable reference genes were selected using the GeNorm applet [1] which calculated a normalization factor. Selected reference genes were *B2m*, *Gapdh* and *Hprt* for mouse and *B2M* and *HPRT* for human. The results were expressed as normalized ratio:  $(C^{\circ} \text{ gene of interest} : C^{\circ} \text{ normalization factor})_{\text{cDNA sample X}} : (C^{\circ} \text{ gene of interest} : C^{\circ} \text{ normalization factor})_{\text{reference cDNA}}$ .

### Proteome study of mouse plasma

*Mouse sample preparation:* Plasma samples were albumin, transferrin and IgG depleted by using the multiple affinity removal spin cartridge-mouse 3 (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's instructions. Sample proteins were then precipitated in trichloroacetic acid 10% (v/v), centrifuged and the pellet resuspended in rehydration buffer (8M urea, 2% w/v CHAPS, 40mM DTT, 0.5% IPG buffer (4-7 ampholytes) and Bromophenol Blue (trace, Bio-Rad, Hercules, CA, USA).

*2D-electrophoresis:* Two-dimensional electrophoresis (2-DE) was carried out using the IPGphor Isoelectric Focusing System and the Ettan dalt six electrophoresis system (Amersham Biosciences, Little Chalfont, UK). For the first dimension analysis, 150 µg of proteins were loaded onto IPG strips (linear pH 4–7 gradient, GE Healthcare, Little Chalfont,

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3 UK) for passive in-gel rehydration (6h at 20°C) and separated by isoelectrofocalisation (IEF).  
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5 Following equilibration steps, IPG strips were sealed on top of priorly polymerized gels  
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7 (12.5% duracryl) for second dimension proteins separation. The gels were then fixed and  
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9 protein spots in gels were visualized by silver staining.  
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13 *Computer analysis of 2-DE patterns and proteins identification:* Images of gels were digitized  
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15 with a calibrated densitometer (GS-800, Bio-Rad, Hercules, CA, USA) and protein patterns  
16  
17 were analyzed using the SameSpots Progenesis Software (nonlinear dynamics, Hospital Vall  
18  
19 d'Hebron, Barcelona, Spain). Proteins of interest were manually excised and digested in-gel  
20  
21 using a 96-well ZipPlate kit for sample preparation (Millipore Ibérica SA Madrid, Spain).  
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23 Proteins were reduced, alkylated, and digested with sequence grade trypsin (Promega,  
24  
25 Madison, WI, USA) according to the manufacturer's protocol. Peptides were eluted in 60%  
26  
27 v/v ACN/ 0.1% v/v TFA and proteins were then identified either by peptide mass  
28  
29 fingerprinting (MALDI-TOF MS, Voyager DE-PRO in reflectron mode, Applied Biosystems,  
30  
31 Foster City, CA, USA) or by peptide sequencing using nano(n)ESI-IT MS/MS (Finnigan  
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33 LCQ IT mass spectrometer, ThermoQuest, Finnigan MAT, San Jose, CA, USA), equipped  
34  
35 with a nanospray source (Protana, Odense, Denmark). MASCOT (Matrix Science, London,  
36  
37 UK) and the Protein Prospector v 3.4.1 (UCSF Mass Spectrometry Facility, University of  
38  
39 California) were used for subsequent protein identification.  
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44 Swiss-Prot (European Bioinformatics Institute, Heidelberg, Germany, Update 02/20/02),  
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46 MSDB, and NCBI nr databases were used for the search.  
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#### 50 **Parenchymal iron determination in mouse liver samples**

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52 The liver iron content was determined by acid digestion of tissue samples as described by  
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54 Torrance and Bothwell [2] followed by iron quantification. Iron from tissue lysates was  
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3 measured with an Olympus AU400 clinical chemistry analyzer (Beckman Coulter, Brea, CA,  
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5 USA) using the human iron assay kit.  
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### 9 **Western-blot analysis of mouse and human liver samples**

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11 Crude membrane fractions from homogenized tissues were prepared as followed; livers were  
12  
13 frozen in liquid nitrogen immediately after removal, and were homogenized using a glass  
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15 potter with a tight fitting teflon pestle rotated at 1,500 revolutions/min in 10 mL/g of tissue of  
16  
17 a solution consisting in 0.25 M sucrose/0.03 M histidine (pH 7.2) supplemented with 2 mM  
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19 EDTA, 0.1 mg/mL phenylmethylsulfonyl fluoride, 2 µg/mL leupeptin, 1 µg/mL pepstatin, and  
20  
21 2 µg/mL aprotinin. The homogenate was then centrifuged at 6,000g for 15 minutes. The  
22  
23 supernatants were then centrifuged at 45,000g for 60 minutes and the pellet was resuspended  
24  
25 in sucrose/histidine buffer and stored frozen at -80°C until use. Protein concentration in the  
26  
27 various membrane fractions was determined with the Bradford assay (Bio-Rad, Hercules, CA,  
28  
29 USA).  
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33 For each lane, 30 µg of protein were extracted and solubilized in 1X Laemmli buffer and were  
34  
35 incubated for 30 min at RT prior to SDS-PAGE electrophoresis and electro-transfer onto a  
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37 polyvinylidene fluoride (PVDF) membrane. To control loading and transfer, membranes were  
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39 stained with Ponceau red after transfer, and subsequently preincubated with blocking solution  
40  
41 (7% skim milk in TBST (0.15% Tween 20, in Tris buffered saline)). Membranes were then  
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43 incubated overnight at 4°C with the following primary antibodies: HO1 (1/6,000; Stressgen,  
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45 San Diego, CA, USA), ALAS1 (1/2,000; Abcam, Cambridge, UK) and β-actin (1/10,000;  
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47 Sigma-Aldrich, Saint-Louis, MO, USA). After washing with TBST, blots were incubated with  
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49 goat peroxidase-labeled anti-rabbit IgG (1/10,000; Jackson ImmunoResearch, West Grove,  
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51 PA, USA) or with sheep peroxidase-labeled anti-mouse IgG (1/5,000; Jackson  
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53 ImmunoResearch, West Grove, PA, USA) for 1 h at RT and underwent chemiluminescent  
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3 detection using Luminata™ Forte Western HRP Substrate (Millipore, Billerica, MA, USA).  
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5 Protein expression was then quantified by densitometry.  
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### 9 **Cytokine determination in mouse liver samples**

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11 Frozen liver sample were disrupted and homogenized with a rotor stator homogenizer, in the  
12 presence of lysis buffer containing 100 mM Tris pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM  
13 EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, Complete Protease Inhibitor Cocktail  
14 Tablets (Roche, basel switzerland) and 1 mM aminoethyl benzenesulfonyl fluoride. The  
15 homogenate was centrifuged at 6,000g for 20 min at 4°C. The supernatant was simultaneously  
16 analyzed for the levels of 10 mouse cytokines and chemokines using the MILLIPLEX® MAP  
17 Mouse Cytokine/Chemokine Magnetic Bead Panel (Millipore, Billerica, MA, USA),  
18 according to the manufacturer's instructions. The 10 cytokines and chemokines analyzed  
19 included Ifn- $\gamma$ , Il-1 $\beta$ , Il-4, Il-6, Il-10, Il-12 (p40), Il-13, monocyte chemoattractant protein 1  
20 (Mcp-1), macrophage inflammatory protein 1 $\alpha$  (Mip-1 $\alpha$ ) and Tnf- $\alpha$ . The assays were  
21 performed with a Luminex technology instrument using Xponent software (Luminex, Austin,  
22 TX). Standard curves were generated for each cytokine/chemokine using standards included  
23 in each kit.  
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### 42 **Pathologic examination of liver samples**

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44 Representative paraffin-embedded blocks of liver tissue were selected for histological  
45 analyses and immunohistochemical studies. A pathological review was performed on  
46 hematoxylin & eosin and Perls stained sections. The immunodetection of MPO (1:500,  
47 polyclonal, Dako, Agilent Technologies, Santa Clara, CA, USA), CD68 (1:500, clone KP1,  
48 Dako, Agilent Technologies, Santa Clara, CA, USA) and CD206 (1:100, clone 5C11, Acris,  
49 Origene, Rockville, MD, USA) was performed on serial sections using an automated  
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immunohistochemical stainer according to the manufacturer’s guidelines (streptavidin-  
peroxidase protocol, BenchMark, Ventana, Roche, Basel Switzerland). A semi-quantitative  
scoring was used for all markers.

**Statistical analysis**

Non parametric Mann-Whitney tests were performed using GraphPad Prism V4 software  
(GraphPad Software, La Jolla, CA, USA).

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## References

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2. Torrance, J. D. and Bothwell, T. H. (1968) A simple technique for measuring storage iron concentrations in formalinised liver samples. *S Afr J Med Sci* **33**, 9-11

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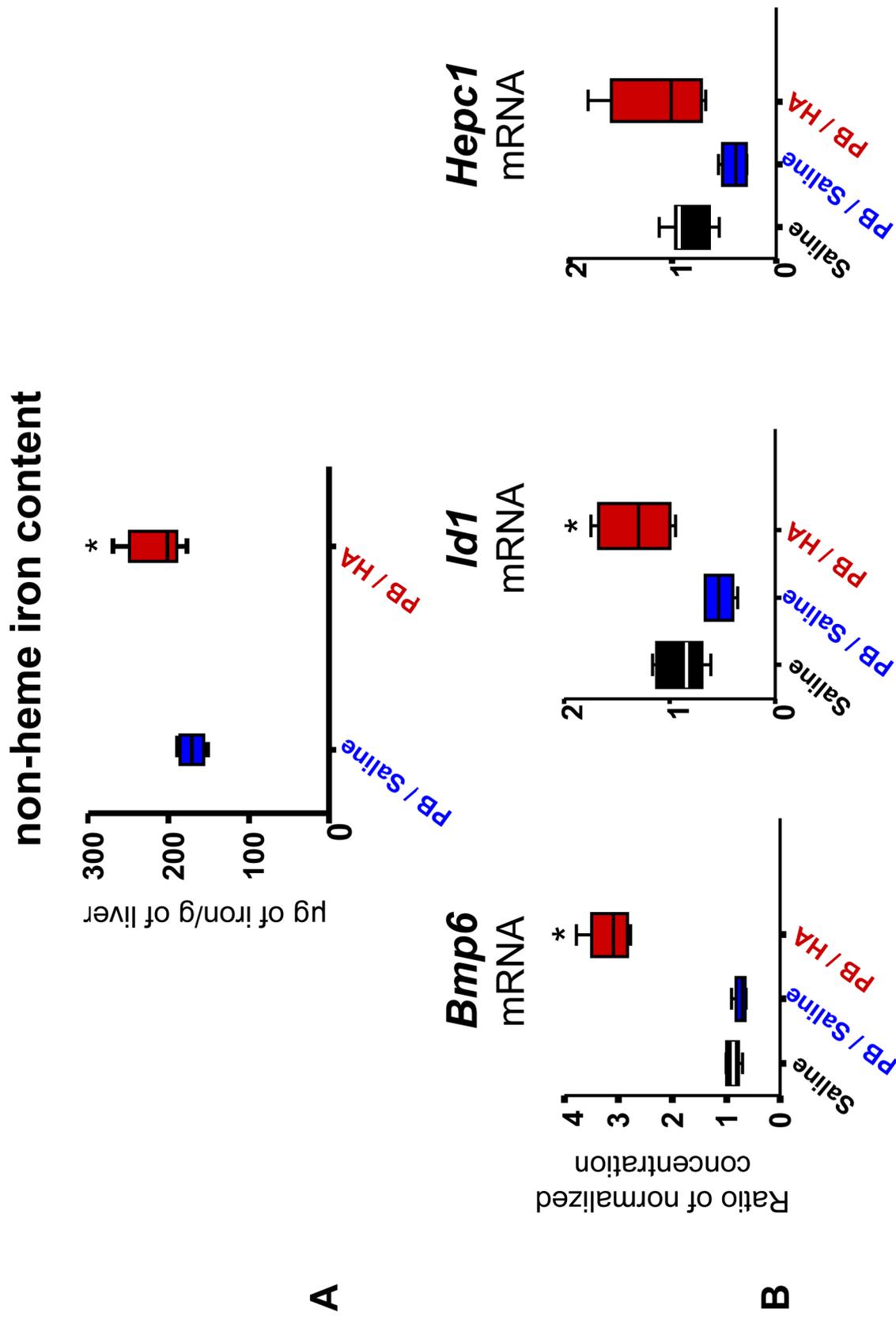


Figure S1