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Pentosan polysulfate to control hepcidin expression *in vitro* and *in vivo*

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ABSTRACT

Hepcidin peptide is crucial in the regulation of systemic iron availability controlling its uptake from the diet and its release from the body storage tissues. Hepcidin dysregulation causes different human disorders ranging from iron overload (e.g. hemochromatosis) to iron deficiency (e.g. anemia). Hepcidin excess is common in the Anemia of Chronic Diseases or Anemia of Inflammation and in the genetic form of anemia named IRIDA; the pharmacological downregulation of hepcidin in these disorders could improve the anemia. Commercial heparins were shown to be strong inhibitors of hepcidin expression, by interfering with BMP6/SMAD pathway. The non-anticoagulant heparins, modified to abolish the anti-thrombin binding site, were equally potent and could be used to improve iron status. To perform its anti-hepcidin activity heparin needs 2O- and 6O-sulfation and an average molecular weight (MW) up to 4000–8000 Dalton, depending on the sulfation level. The pentosane polysulfate (PPS), which shares with heparin a high degree of sulfation, is a compound with low anti-coagulant activity that is already in use for pharmaceutical treatment. In the present work we analyzed the anti-hepcidin activity of PPS *in vitro* and *in vivo*. We found that it acts as a strong inhibitor of hepcidin expression in HepG2 cells with an effect already visible after 2–3 h of treatment. It also suppressed hepcidin in mice in a dose dependent manner after 3 h and with a significant redistribution of systemic iron without evident side effects. PPS is also able to abolish the LPS dependent hepcidin upregulation similarly to that showed for heparin derivatives. These results suggest PPS as an interesting compound to control hepcidin *in vivo*.

1. Introduction

Hepcidin is a peptide hormone produced by the liver, with an essential role in controlling systemic iron availability. It binds ferroportin, the sole known cellular iron exporter, mediating its internalization and degradation and thus reducing iron release [1,2]. Essential for the hepcidin-ferroportin axis is the regulation of hepcidin expression, which is controlled by various factors, mostly connected with iron abundance and utilization, inflammation, hypoxia and erythropoietic activity [3,4]. Two major pathways are known to be responsible for hepcidin expression. The iron-mediated pathway involves the BMP/SMAD signaling pathway, in which BMP6 is the systemic iron sensing, able to induce liver hepcidin expression. It interacts with its receptors, a complex formed by the dimerization of type-II and type-I BMP-receptor and co-receptor Hemojuvelin (HJV), with subsequent phosphorylation of SMAD1/5/8, association with SMAD4 and translocation to the nucleus [4]. The second pathway involves the inflammatory cytokine IL-6 and JAK/STAT3 pathway [5] which cooperates with BMP/SMAD pathway to stimulate hepcidin expression in condition of inflammation [6]. Hepcidin dysregulation is common in many pathologies connected to iron imbalance. Hereditary hemochromatosis is characterized by low levels of hepcidin with consequent iron overload due to genetic mutation in iron-related genes [7]; “Iron Refractory Iron Deficient Anemia” (IRIDA) a genetic form of anemia, is characterized by high levels of hepcidin and consequent severe iron deficiency anemia due to mutations on Tmprss6 (a serine protease acting on the co-receptor HJV as negative controller of BMP/SMAD pathway) [8–10] and Anemia of Chronic diseases (ACD) or Anemia of inflammation (AI), common forms of anemia caused by chronic diseases, infections and/or inflammation, and characterized by high hepcidin levels and iron deficiency [4]. Due to the relevance of these diseases, many groups studied different pharmacological approaches to modulate hepcidin and restore a proper iron homeostasis [11]. In particular, we found that heparin is a compound that suppresses basal, BMP6- and IL6-induced hepcidin expression, *in vitro* and *in vivo* [12]. Heparin is a highly sulfated glycosaminoglycan, widely used in clinic for its anti-coagulant activity. We

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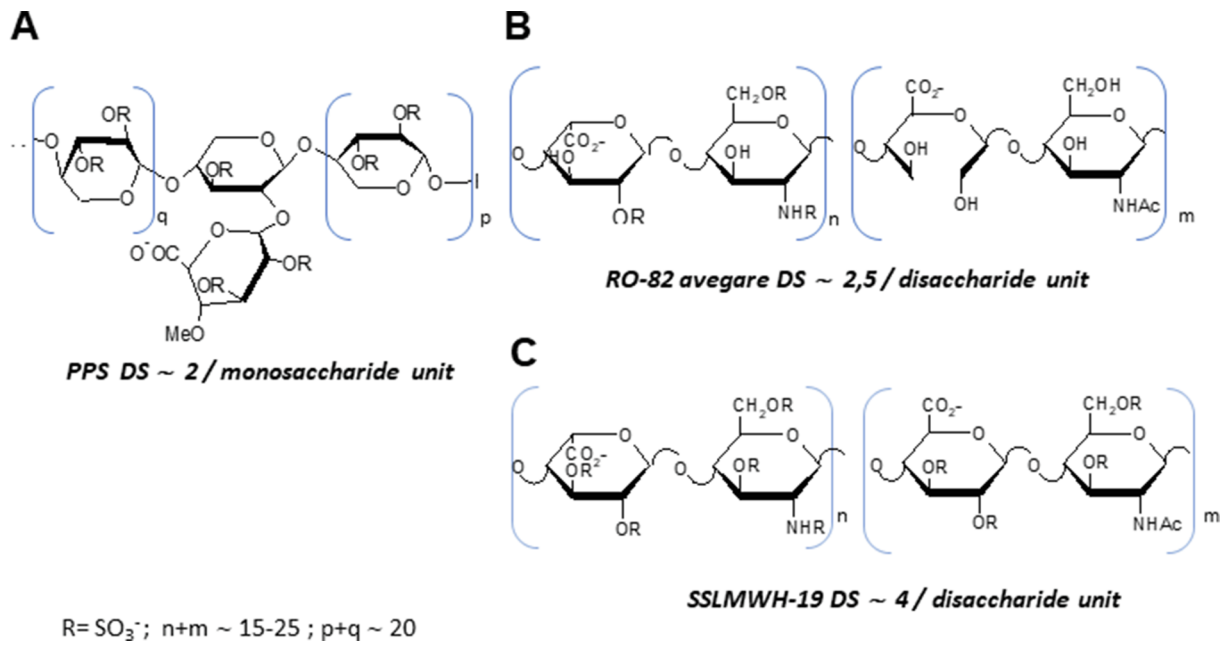

Fig. 1. Structure of PPS, RO-82 and SSLMWH-19. The structure of pentosane polysulfate (PPS, A) is characterized by a sulfation degree (DS) ~ 2 and monosaccharide unit repetition (p + q) ~ 20, glycol-split (RO-82, B) is characterized by a sulfation degree (DS) ~ 2.5 and disaccharide unit repetition (n + m) ~ 25 and oversulfated (SSLMWH-19, C) is characterized by a sulfation degree (DS) ~ 4 and disaccharide unit repetition (n + m) ~ 15. R is the sulfation groups (SO_3^-).

demonstrated that heparins and chemically-modified heparins, devoid of anti-coagulant property, such as the glycol-split and oversulfated heparins, were able to strongly and readily suppress hepcidin expression and BMP6/SMAD pathway in Hepatoma cell lines and also in mice, where we observed not only an efficient modulation of hepcidin but also an iron redistribution, without showing adverse side effects [13,14]. In other studies, we showed that for anti-hepcidin activity the heparins need high level of sulfation (with sulfate groups in position 2-O and 6-O) and a molecular weight up to 7000 Da, depending on the sulfation degree. Specifically heparins with high level of sulfation require a lower molecular weight (about 4000 Da, such as oversulfated heparins), on the other hand normal level of sulfation requires a molecular weight up to 6000–7000 Da such as glycol-split heparins [15].

Pentosan Polysulfate (PPS), due to its high sulfation degree, is considered part of the heparinoid class [16], by sharing a certain number of biological activities with heparin despite its low-molecular weight (4000–6000 Da). It is synthesized by chemical sulfonation process of a beech tree-derived β-(1 → 4)-xylan and this xylan backbone could be also substituted with 4-methyl glucopyranosyl uronic acid units glycosidically linked to the 2-position of the main chain [17] (Fig. 1). Practically all hydroxyl groups of this compound are sulfated, so that its monosaccharide sulfation degree is about 2, much higher than that of heparin and its RO-derivative (e.g. RO-82) (about 2.5 per disaccharide unit, or about 1.2 per monosaccharide) and similar to that of LMW-oversulfated (e.g. SSLMWH-19) heparin (about 4 per disaccharide or 2 per monosaccharide). The natural variability of the starting material and the following modifications, that could be introduced during the synthesis, produce a polydisperse polymer consisting of a set of heterogeneous chains, differing in molecular weight and type/number of substituents [31], but it does not affect its main feature such as the charge density. Regarding the average molecular weight PPS can be compared to the low molecular weight heparins and to the oversulfated heparin previously used for the interaction with BMP [14].

PPS has been used in clinic for different therapeutic purposes. It has been used for years as an anti-thrombotic compound, due to its fibrinolytic activity and anti-coagulant property, even if 15 fold weaker than the commercial anti-coagulant heparins [17]. The commercial available formulation of PPS approved by AIFA (the Italian regulatory authority for drugs) is named Fibrase®, manufactured both as oral and injectable solution and prescribed as anti-thrombotic compound, whereas Elmiron® is approved by FDA, EMA and AIFA as oral treatment of interstitial cystitis (IC) and painful bladder [18]. Interestingly, recent papers suggested the use of PPS for therapy of chronic diseases such osteoarthritis [19,20], prion diseases [21], glomerulosclerosis [22], mucopolysaccharidoses [23,24] and lysosomal storage syndrome such as Fabry and Gaucher Diseases [25], but none of them verified if PPS treatment affected hepcidin levels and iron parameters.

The present work had the aim to verify if PPS has an anti-hepcidin activity in hepatoma cell line and in mice. Our results showed that PPS strongly and rapidly inhibited hepcidin expression suppressing BMP/SMAD pathway both *in vitro* and *in vivo* where we observed also a consequent effect on systemic iron distribution, without causing evident side effects.

2. Materials and methods

2.1. Cell culture and chemical treatment

The human hepatoma cell line HepG2 (Lombardy and Emilia Romagna Experimental Zootechnic Institute, IZSLER) was cultured in minimum essential medium (Gibco from Life technologies) supplemented with 10% endotoxin-free fetal bovine serum (Gibco), 0.04 mg/mL gentamicin (Euroclone), 2 mM L-glutamine (Gibco), and 1 mM sodium pyruvate (Carlo ERBA) and maintained at 37 °C in 5% CO2. HepG2 cells ($3X10^5$ cells/well) were seeded into 12-well plates, and after 24 h, the heparinod compound Pentosan Polysulfate (PPS, which was freeze-dried from vials of fibrase lot G144496 and used as it is) was added at different concentrations (0.004–0.012–0.04–0.12–0.4–1.2–3.6–11 μg/mL), different time points (0–1–2–3–4–6 h) and with or without 10 ng/mL BMP6, 50 ng/mL IL-6 (ReliaTech GmbH), or 2 ng/mL Oncostatin-M (Sigma-Aldrich) for 6 h. As control, cells were treated with (0.004–0.012–0.04–0.12–0.4–1.2–3.6–11 μg/mL) glycol-split (RO-82) or oversulfated (SSLMWH-19) heparins for 6 h, in presence or absence of 10 ng/mL BMP6.

2.2. Quantitative qRT-PCR

After the treatments, total cell RNA was recovered with TRI Reagent (Sigma-Aldrich), according to the manufacturer's instruction. Reverse transcription was performed using 1 μg RNA, oligo dT, and Improm Reverse Transcriptase (Promega) in 20 μL. Samples of 1 to 2 μL were used for quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay, using iTAq Universal SYBR Green (Bio-Rad), according to the manufacturer's instructions. Primers used for human cell lines were HsHamp: for, 5′-CCA-GCTGGA-GC-CCA-TGT-T-3′ and rev, 5′-GCC-GCA-GCA-GAA-AATGCA-3′; HsHprt1: for, 5′-TGC-TTT-CCT-TGG-TCA-GGC-AG-3′ and rev, 5′-AAG-CTT-GCG-ACC-TTG-ACC-AT-3′; HsId1: for, 5′-GTA-AAC-GTG-CTG-CTC-TAC-GAC-ATG-A-3′ and rev, 5′-AGCTCC-AAC-TGA-AGG-TCC-CTG-A-3′. The same procedure was used for mouse liver and the primers for quantitative real-time RT-PCR assay were: MmHamp1: for, 5′-AAG-CAG-GGC-AGA-CAT-TGC-GAT-3′, and rev, 5′-CAG-GAT-GTG-GCT-CTA-GGC-TAT-GT-3′; MmHprt1: for, 5′-CTG-GTT-AAG-CAG-TAC-AGC-CCC-AA-3′ and rev, 5′-CAGGAG-GTC-CTT-TTC-ACC-AGC-3′; MmId1 for, 5′-ACC-CTG-AACGGC-GAG-ATC-A-3′ and rev, 5′-TCG-TCG-GCT-GGA-ACA-CAT-G-3′; MmSocs3: for, 5′-TTA-AAT-GCC-CTC-TGT-CCC-AGG-3′ and rev, 5′-TGT-TTG-GCT-CCT-TGT-GTG-CC-3′;

2.3. Immunoblots

Total protein extracts were prepared incubating cells in lysis buffer (200 mM Tris-HCl at pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 10% glycerol, 1 mM sodium fluoride, 1 mM sodium orthovanadate; Complete Protease Inhibitor Cocktail; Sigma). Protein content was determined by colorimetric assay (MicroBCA Protein Assay Kit, Thermo Scientific) and 50 mg of total protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to HYBOND-P membrane (GE). Membranes were probed with antiphospho-SMAD5 (Cod. AB92698 Abcam), SMAD5 (Cod. 9517, Cell Signaling), pSTAT3 (Cod. ABP-4001, Immunological Sciences), STAT3 (Cod. SC-482, Santa Cruz), GAPDH (Cod. SC-166574, Santa Cruz) and Actin (Cod. TA890010, OriGene Technologies) antibodies and then developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and visualized on CL-XPosure Film (Thermo Scientific).

2.4. Mice

C57BL/J6 male mice (Harlan Laboratories) were kept on a standard diet until 8 or 9 weeks. The study was approved by the Institutional Animal Care and Use Committee of the University of Brescia, Italy. Four or five mice per experimental group were used for each type of experiment performed.

a) In the first experiment, the mice were treated subcutaneously (SC) with saline or PPS (single dose) and euthanized at different time points. Blood was collected for serum hepcidin and serum iron evaluation. Liver and spleen were analyzed for mRNA, protein, and/or iron content.
b) In the second experiment, the mice were treated subcutaneously (SC) with saline or different doses of PPS and euthanized 6 h after the treatment.
c) In the third experiment, the mice were treated intraperitoneally (IP) with LPS (1 mg/kg, to simulate an acute inflammation status) and subcutaneously (SC) with PPS. Six hours after the treatment they were euthanized and analyzed as described earlier.
d) In the fourth experiment the mice were treated subcutaneously (SC) or orally (by gavage) with saline or different doses of PPS to compare the two different way of treatment. Six h after the treatment, blood was collected for serum hepcidin and serum iron evaluation, and the mice were euthanized. Liver and spleen were analyzed for mRNA, protein, and/or iron content.

Spleen iron content was determined spectrophotometrically as in Roetto et al, with minor modifications [30]. Serum iron was determined spectrophotometrically with a commercial kit, according to the manufacturer's instruction (Randox Laboratories).

2.5. Serum hepcidin determination by mass spectrometry

Hepcidin-25 was measured with a mass spectrometry-based method included in an international harmonisation study [29]. Hepcidin-25 standards (native and the isotopic-labelled internal standard [Asp-Thr-His-[13C9,15 N]Phe-Pro-Ile-Cys-Ile-[13C9,15 N]Phe-Cys-Cys-[15 N]Gly-Cys-Cys-His-Arg-Ser-Lys-Cys-Gly-Met-Cys-Cys-Lys-Thr] (Mr2810.2)) were purchased from Peptide International (Louis-ville, KY, USA); 50 μL was used for each serum, and calibrants and blank serum (deprived from hepcidin by charcoal treatment) were used as reference. Samples were treated by solid-phase extraction using Oasis hydrophilic-lipophilic balanced reversed-phase (HLB) cartridges (Waters, Milan, Italy). HPLC was performed using an X-Terra MS C182.5 lm (Waters), and detection was obtained using a Triple Quad LC-MS/MS (Agilent Technologies, Santa Clara, CA, USA).

2.6. Statistics

Data are shown as mean ± standard deviation (± SD) as indicated in the figures. Generally, expression levels were scaled to control values and expressed as fold change for *in vitro* experiments and as -dCt for *in vivo*. Comparison of values between untreated or treated cells as well as comparison between saline or Pentosan Polysulfate (PPS) treated mice were performed by one-way ANOVA. Multiple comparisons were corrected by Tukey's test (GraphPad Prism6, GraphPad Software). Differences were defined as significant for P values < 0.05 and p-values reported on the graphs or in the figure legends. In figure 2 the symbols *; **; ***; **** represent a P value of $p \leq 0.05$; $p \leq 0.001$; $p \leq 0.0005$; $p \leq 0.0001$ respectively. In figure 3 the symbol ▵ represents a P value of $p \leq 0.0001$.

3. Results

3.1. PPS inhibited hepcidin expression in HepG2 cells

Heparin derivatives are known to suppress hepcidin expression *in vitro* and *in vivo*, offering an interesting strategy to control its expression. The structure of PPS in comparison with that of heparin, differs for the sugar backbone, molecular weight and for the higher charge density but comparable to that of the, previously studied, oversulfated heparin (SSLMWH-19), and this prompt us to investigate the effect of PPS on hepcidin expression. First, we analyzed its activity on basal and BMP6-induced hepcidin expression in HepG2 cells, therefore different concentrations of PPS (0.004–0.012–0.04–0.12–0.4–1.2–3.6–11 μg/mL) were used to treat the cells with or without the presence of BMP6 (10 ng/mL) and the level of hepcidin mRNA was analyzed after 6 h of treatment. As control, we used glycol-split (RO-82) and the oversulfated (SSLMWH-19) heparins at the same weight concentrations of PPS. We found that PPS suppressed the basal level of hepcidin at concentrations as low as 0.12–0.4 μg/mL with an inhibition of about 80–90% and it showed a stronger anti-hepcidin activity than RO-82 and SSLMWH-19 with about 15% and 20–40% hepcidin suppression with RO-82 and SSLMWH-19, respectively (Fig. 2A). In presence of BMP6 (10 ng/mL) PPS suppressed the hepcidin upregulation in a dose dependent manner with a total suppression of BMP6 induction at 0.12–0.4 μg/mL (Fig. 2B), showing a stronger activity compared to RO-82 and SSLMWH-19. As previously published, heparins suppressed hepcidin expression acting on BMP6/SMAD pathway likely interfering with some components of the membrane complex (such as BMP6, receptors or co-receptor). To verify if also the anti-hepcidin activity of PPS is due to the suppression of BMP6/SMAD pathway, we treated HepG2 cells with

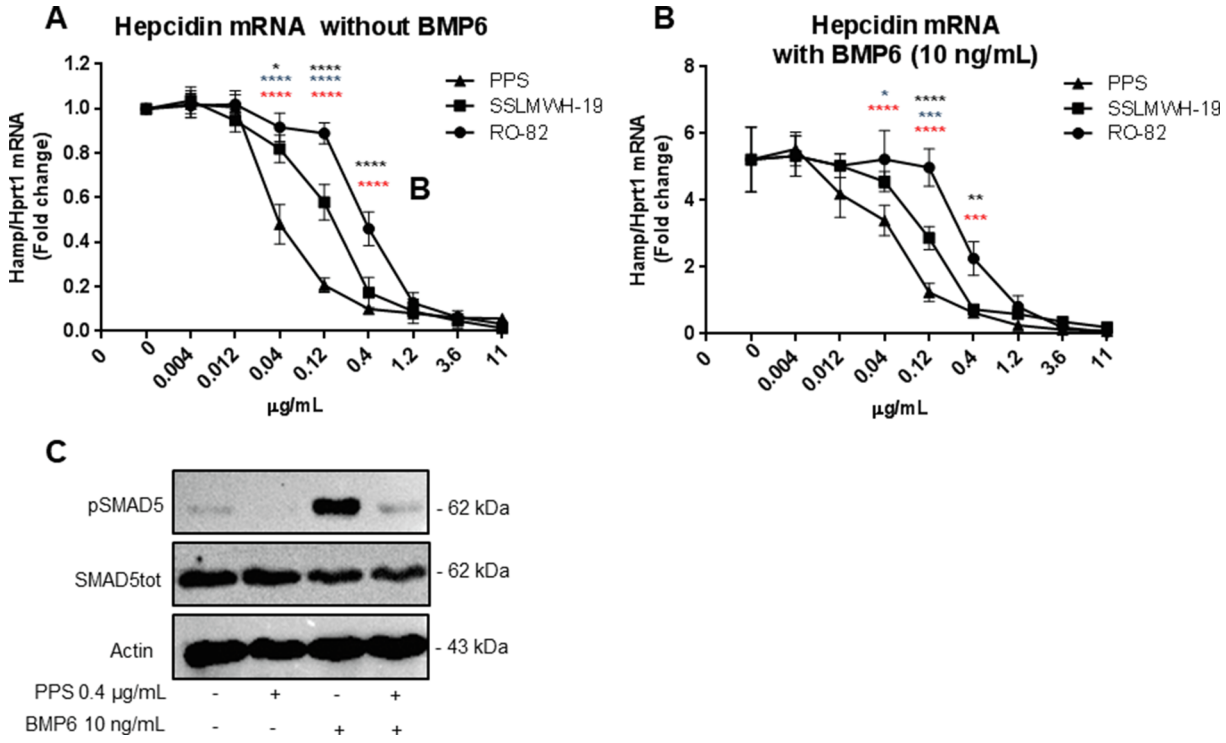

Fig. 2. PPS reduced basal and BMP6-induced hepcidin expression. HepG2 cells were treated with PPS, RO-82 and SSLMWH-19 at different concentrations (0.004, 0.012, 0.04, 0.12–0.4–1.2–3.6–11 µg/mL) without BMP6 (A) or in presence of 10 ng/mL BMP6 (B) for 6 h. Hepcidin mRNA was quantified with qRT-PCR in relationship to Hprt1 mRNA. Data are shown as means and SD of 3 different experiments and expressed as fold change and expressed as fold change in comparison to the basal level of untreated cells (0). Red asterisks are the statistical significance between PPS and RO-82, the blue asterisks are that between PPS and SSLMWH-19 and black asterisks are that between SSLMWH-19 and RO-82 (C) Western blotting analysis of pSMAD5, SMAD5tot and Actin (as calibrator of the cell extracts) after the incubation with PPS (0.4 µg/mL) with or without BMP6 (10 ng/mL) for 6 h. The image is representative of 3 independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

0.4 µg/mL PPS with or without BMP6 (10 ng/mL) for 6 h and then we analyzed the level of phosphorylated SMAD5 compared to total SMAD5, with actin as loading control. PPS totally abolished the basal phosphorylation of SMAD5 without interfering with the total level of the protein and interestingly in presence of BMP6 (10 ng/mL) that induced a significant increase of pSMAD5, PPS strongly suppressed BMP6-induced phosphorylation (Fig. 2C), suggesting an inhibitory mechanism similar to that of heparins.

3.2. PPS inhibited hepcidin expression in HepG2 cells after 3 h

Depending on the molecular weight of heparin derivatives, we showed that the inhibition of hepcidin in cells takes place starting from 3 h (e.g. oversulfated heparin, [14]) or 6 h (e.g. glycol-split heparin, [13]). To verify the time-dependent down regulation of hepdicin, due to PPS treatment, we analyzed the time course of hepcidin inhibition in HepG2 cells using 0.4 µg/mL PPS. It inhibited basal hepcidin expression of about 50% after 3 h of treatment (Fig. 3). As already known, BMP6 induction of hepcidin starts after 1 h and increases until at least 6–12 h. We found that BMP6 (10 ng/mL) induced an increase of hepcidin mRNA with about 2, 4 and 8 fold-change after 2, 4 and 6 h respectively, compared to the starting point (0 in Fig. 3, black line), as expected the co-treatment BMP6 (10 ng/mL) and PPS (0.4 µg/mL) strongly suppressed the BMP6-activation of hepcidin that started to be significant after 3 h of co-treatment (Fig. 3). Thus, the anti-hepcidin activity of PPS in HepG2 cells is fast and persists for a long time (at least up to 6 h).

3.3. PPS reduced hepcidin induction by inflammatory stimuli in HepG2 cells

The alteration of SMAD pathway occurs also on inflammation dependent hepcidin regulation, in fact without the support of an active

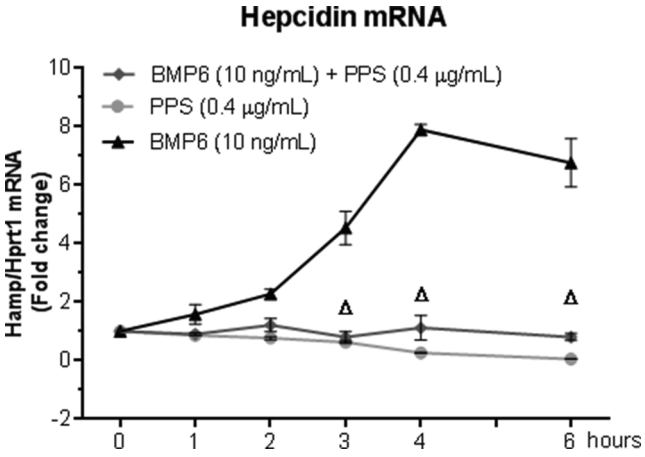

Fig. 3. PPS reduced BMP6-depepndent hepcidin expression in HepG2 in a time dependent manner. HepG2 cells were treated with 0.4 µg/mL PPS and the level of hepcidin mRNA evaluated with qRT-PCR in relationship to Hprt1 mRNA, at different time of exposure (1–2-3–4-6 h) without or with BMP6 (10 ng/mL). The values are expressed as fold change over the untreated cells (0). Data are shown as means and SD of 3 different experiments. The triangles are the statistical significance between BMP6 and BMP6 plus PPS.

BMP/SMAD pathway, STAT3 is not able to upregulate hepcidin [6]. We also showed that heparins reduce basal, BMP6- and IL6-dependent hepcidin expression affecting the BMP6/SMAD pathway without interfering with JAK/STAT3 pathway. To verify this point with PPS, HepG2 cells were treated with 0.4 µg/mL PPS with or without two different inflammatory stimulators, IL6 (50 ng/mL) and Oncostatin-M (2 ng/mL). As shown previously, PPS significantly suppressed basal

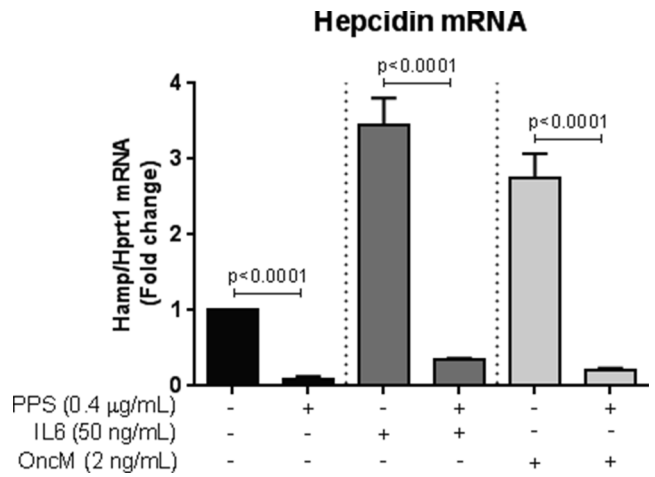

Fig. 4. PPS reduced IL-6 and Oncostatin-M induced hepcidin mRNA. HepG2 cells were treated for 6 h with 0.4 µg/mL PPS alone (black histograms) or in combination with 50 ng/mL IL-6 (dark grey histograms) or 2 ng/mL Oncostatin-M (OncM) (light grey histograms). Hepcidin mRNA levels were quantified with qRT-PCR in relationship to Hprt1 mRNA. The values are presented as means and SD of 3 different experiments and are expressed as fold change over the untreated cells.

hepcidin mRNA after 6 h treatment (black histograms, Fig. 4). IL6 induced an increase of hepcidin expression of about 3.5-fold in 6 h but in presence of 0.4 µg/mL PPS it is not able to be effective (dark grey histograms in Fig. 4). The same behavior was observed for Oncostatin-M (OncM) that increased hepcidin of about 2.6–3-fold in 6 h when it is alone but was completely ineffective in the presence of 0.4 µg/mL PPS (light grey histograms in Fig. 4). The results suggest that PPS, such as heparin derivatives, is able to control both iron- and inflammation-dependent hepcidin expression.

3.4. PPS reduced hepcidin in mice after 3 h

Taking advantage of the *in vitro* results, we analyzed the time dependent effect of PPS in mice. Healthy male mice (8–9 weeks old) were treated subcutaneously with PPS (20 mg/kg) (a dose we used for other heparin derivatives) and they were euthanized after 3 and 6 h. The level of both mRNA and serum hepcidin was strongly suppressed at 3 h and also up to 6 h (Fig. 5 A and B). To verify the suppression of pSMAD pathway, we analyzed the level of Id1 that, as expected, was found significantly reduced (Fig. 5C). The reduction of hepcidin and Id1 mRNA is supported by the inhibition of SMAD5 phosphorylation in presence of PPS (Fig. 5G). The treatment was effective not only on hepcidin level, but it induced a change in iron distribution with an upward tendency in serum iron (statistically significant after 6 h) (Fig. 5D) and a statistically significant reduction of spleen iron content (Fig. 5E). The liver iron content showed a similar trend but not statistically significant due to variability intragroup (Fig. 5 F) and the ferritin iron did not change significantly (not shown) possibly due to the short-time treatment.

3.5. PPS readily reduced liver hepcidin expression in a dose dependent manner in vivo

As previously showed, PPS at a dose of 20 mg/kg suppressed about 90% of hepcidin in mice after 3–6 h (Fig. 5). To study its dose dependent effect, we injected PPS at doses in the range of 1–20 mg/kg and euthanized the animals after 6 h. At the lowest dose of 1 mg/kg the liver hepcidin mRNA was significantly reduced compared to the control mice (0) (Fig. 6A) with a concomitant reduction of serum hepcidin (Fig. 6C). Increasing the doses of PPS, hepcidin mRNA and serum protein decreased with an inhibition of about 40% (at 5 and 10 mg/Kg) and 50% (at 20 mg/kg) for mRNA level (expressed as -dCt) (Fig. 6A) and a reduction of the protein of about 10 fold at 10 mg/kg, reaching undetectable values with 20 mg/kg PPS (Fig. 6C). Also, the level of Id1 mRNA was reduced in a dose dependent manner in agreement with the reduction of hepcidin expression (Fig. 6B). Their suppression caused by PPS is in agreement with the reduction of pSMAD5 compared to SMAD5tot (Fig. 6G). Serum iron increased in a dose-dependent manner starting from 10 mg/kg (Fig. 6D), and spleen iron was significantly reduced at the dose ≥ 5 mg/kg (Fig. 6E). Liver iron content showed a downward tendency with the highest dose of PPS (20 mg/kg) (Fig. 6F).

3.6. PPS in chemically induced inflammation

To induce an inflammatory response, male mice (8–9 weeks old) (5 animals per experimental group) were treated with an intraperitoneal injection of LPS (1 mg/kg), together with a subcutaneous injection of saline or PPS (5 mg/kg or 20 mg/kg); the mice were euthanized 6 h later. LPS treatment induced a significant increase of hepcidin both as mRNA and serum protein (Fig. 7A and B), as expected. Interestingly, LPS-dependent upregulation of hepcidin was totally abolished in presence of PPS (20 mg/kg) (Fig. 7A and B) although the inflammation marker, Socs3 mRNA level, was equally upregulated in all the LPS groups with or without PPS (Fig. 7C). PPS did not affect the STAT3 phosphorylation as shown in Fig. 7G, whereas it caused a significant reduction in pSMAD5 (Fig. 7G) significant after 20 mg/kg of PPS, supporting the hypothesis that it acts interfering with BMP6/SMAD pathway. The LPS induced reduction of serum iron (Fig. 7D, triangles versus circles), due to the increase of hepcidin expression, as expected (Fig. 7A, triangles versus circles). The treatment with 20 mg/kg of PPS, blocking the upregulation of hepcidin (Fig. 7A), is able to reduce the iron retention in spleen and liver (due to LPS-hepcidin induction) (Fig. 7E and F) which in turn limits the reduction of serum iron (Fig. 7D). Even if these three parameters of systemic iron availability (serum, liver and spleen iron content) showed only a tendency, they are all in line with the anti-hepcidin property of PPS in presence of an acute inflammatory stimulation.

3.7. Oral PPS-treatment

PPS is clinical administered by intravenous or intramuscular injections or by oral administration for some indications, such as for interstitial cystitis, indicating that it can be absorbed from the diet. In our previous paper we administered heparin derivates subcutaneously, but with PPS it was also interesting to verify the effect on hepcidin expression and iron availability after its oral administration in mice. Male mice (8–9 weeks old) (4 animals per group) were treated with a subcutaneous injection of 5 or 20 mg/kg PPS, in parallel with oral gavage of 100–200–300 mg/kg PPS and the mice were euthanized after 6 h of treatment. The subcutaneous injection of PPS reduced hepcidin mRNA and serum protein at the two concentrations tested (Fig. 8A and C), confirming the results presented in Figs. 5, 6 and 7. Id1 mRNA decreased to about 50% (Fig. 8B) and serum iron increased with the dose of 20 mg/kg (Fig. 8D), as in Figs. 5D and 6D. Interestingly, also the oral administration of PPS reduced hepcidin expression and induced an iron redistribution. The dose of 300 mg/kg of PPS reduced hepcidin mRNA of about 50% (Fig. 8A) and serum protein of about 20fold (Fig. 8C). The Id1 mRNA level showed a similar trend (Fig. 8B) in agreement with the reduction of hepcidin expression while serum iron level tended to increase with the high dose of 300 mg/kg of PPS (Fig. 8D). This is the first time that the oral administration of a sulfate polysaccharides is effective in controlling hepcidin expression and iron availability, opening the door for the use of oral administration of PPS to control hepcidin.

4. Discussion

Hepcidin is the master regulator of iron availability controlling both

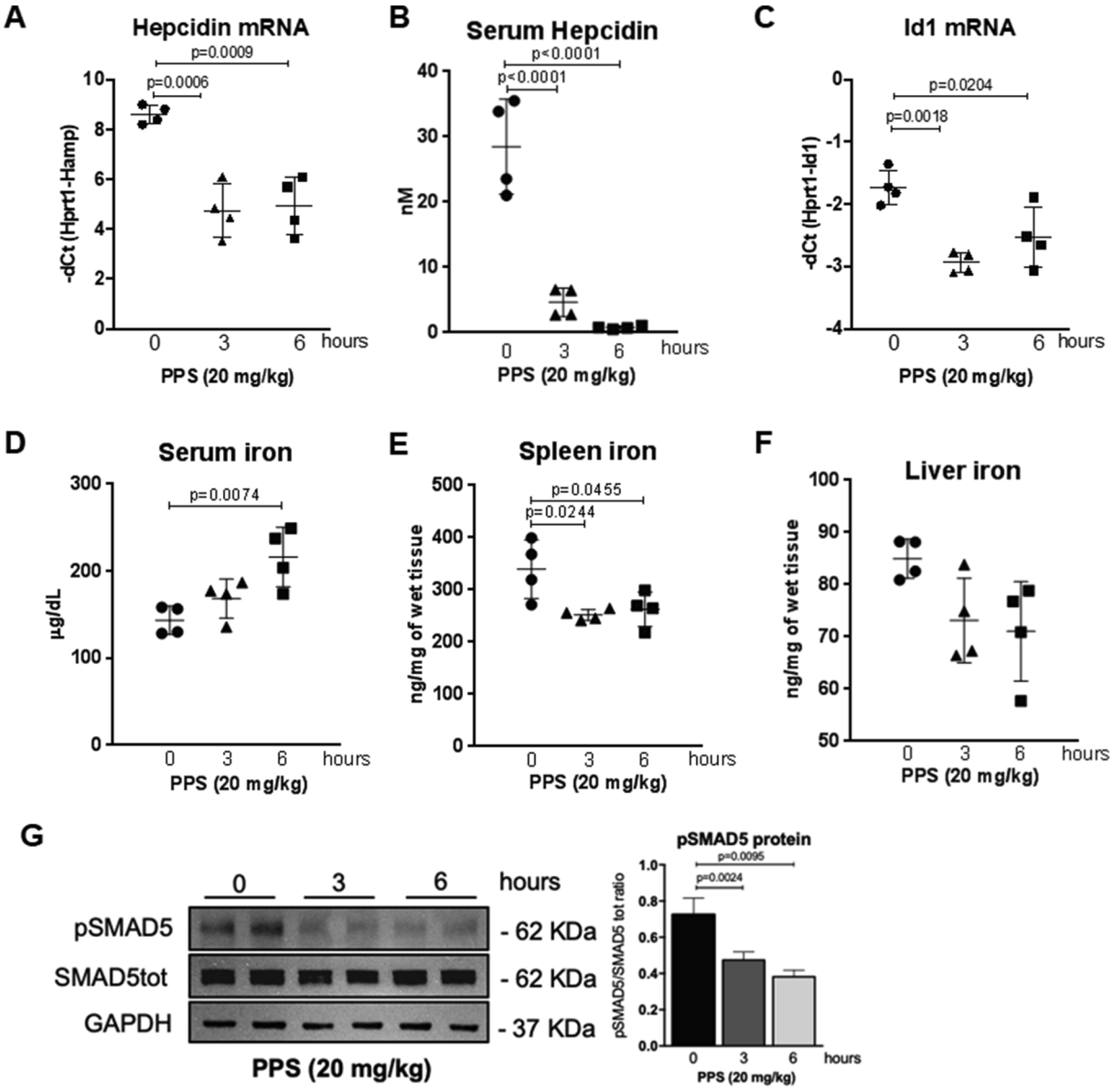

Fig. 5. Single subcutaneous injection of PPS strongly inhibited liver hepcidin and increased serum iron in 3–6 h. Mice (8–9 weeks old) (4 mice per experimental group) were treated subcutaneously (SC) with a single injection of PPS 20 mg/kg or PBS (as control) and euthanized after 3 and 6 h. (A) Hepcidin and (C) Id1 mRNA was quantified by qRT-PCR in relationship to Hprt1 mRNA and expressed as -dCt (Hprt1-Hamp) or -dCt (Hprt1-Id1). (B) Serum Hepcidin was measured by mass spectrometry. (D) Serum iron was measured by commercial kit. (E) Spleen and (F) Liver iron content was measured spectrophotometrically. (G) Western blotting analysis of pSMAD5, SMAD5tot and GAPDH (as calibrator of the samples). The graph showed the densitometry of pSMAD5/SMAD5tot comparing the untreated mice (0) to the treated ones (3 and 6 h).

the iron uptake from the duodenum and its release from iron storage tissues [6]. The dysregulation of hepcidin levels causes some disorders due to iron excess if hepcidin is too low, and, on the contrary, iron deficiency if hepcidin is too high [2]. In the first situation the main groups of disorders are the hemocromatosis (type I, II and III) and, to date, the available treatments include phlebotomies and iron chelation therapy [7].

The opposite situation is due to high level of hepcidin that causes different type of anemia and it is important to reduce the hepcidin expression restoring the correct level of hemoglobin and solve the anemia. Among the different strategies actually under study to reduce hepcidin there is the use of heparin derivatives [4,26]. Heparin derivatives are very potent inhibitors of hepcidin *in vitro* and *in vivo* and their anti-hepcidin activity involves the suppression of BMP6/SMAD pathway with the binding of some components of the membrane complex (BMP6, receptors, co-receptor). Interestingly, the pentosan polysulfate (PPS) (a semi-synthetic sulfated polysaccharide with a molecular weight of about 4000–6000 Da) seems a promising compound to control hepcidin. It is a polymer of xylose and contains about two sulfate groups per carbohydrate monomer. It retains a low anticoagulant and fibrinolytic property, 15 times less than that of heparin and interestingly, it binds different heparin-binding growth factors such as FGFs as well as the receptor FGFR-1. It is already used in human to treat the interstitial cystitis (orally administration) and as a mild antithrombotic (with oral, subcutaneous and intravenous administration). In light of these evidences, PPS could be a promising compound to control hepcidin expression. To verify this hypothesis, we analyzed the effect of PPS in cells (HepG2) and *in vivo* as we previously did to test heparins [12–14].

PPS is functional in suppressing hepcidin expression in HepG2 cells at low concentrations (0.4 µg/mL) and fast (3–6 h), in basal, BMP6- and IL6/OncM-stimulated hepcidin. In the mice, subcutaneous injections of

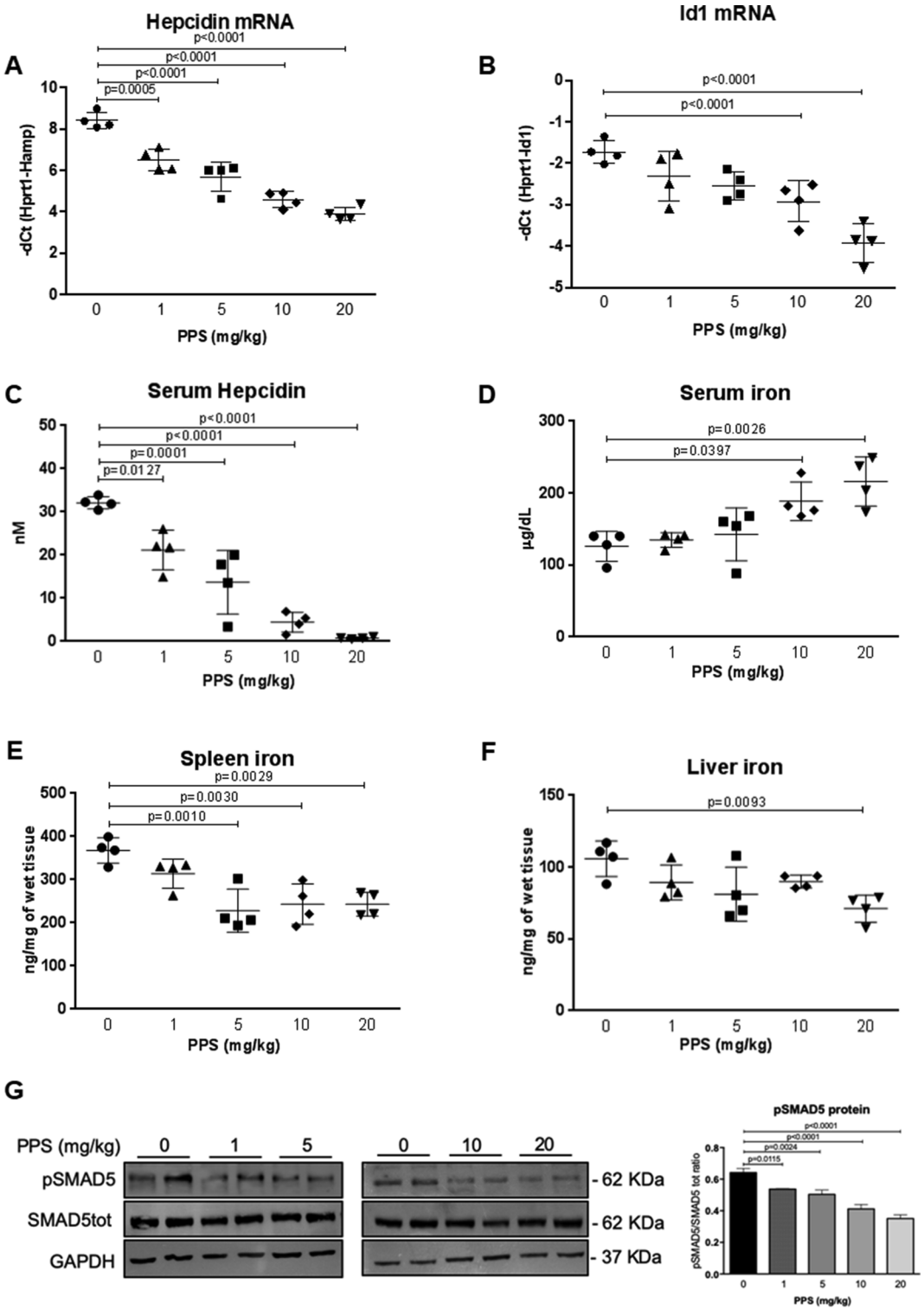

Fig. 6. Single subcutaneous injection of PPS inhibited liver hepcidin in a dose dependent manner. Mice (8–9 weeks old) (4 mice per experimental group) were treated subcutaneously with a single injection of different doses of PPS (1–5–10–20 mg/kg) or PBS (as control) and euthanized after 6 h. (A) Hepcidin and (C) Id1 mRNA was quantified by qRT-PCR in relationship to Hprt1 mRNA and expressed as -dCt (Hprt1-Hamp) or -dCt (Hprt1-Id1). (B) Serum Hepcidin was measured by mass spectrometry. (D) Serum iron was measured by commercial kit. (E) Spleen and (F) Liver iron content was measured spectrophotometrically. (G) Western blotting analysis of pSMAD5, SMAD5tot and GAPDH (as calibrator of the samples). The graph showed the densitometry of pSMAD5/SMAD5tot comparing the untreated mice (0) to the treated ones (1–5–10–20 mg/kg of PPS).

PPS caused a total suppression of hepcidin after 3–6 h. It showed a dose dependent activity (starting to be significant at 5 mg/kg) and it is also effective in suppressing hepcidin in inflammation induced by LPS acting mainly on BMP/SMAD pathway. PPS showed a stronger potency in suppressing hepcidin in cells being effective starting from 0.04 µg/mL compared to glycol-split and oversulfated heparins that, to reach a similar inhibition, require a 10-times higher dose (> 0.4 µg/mL). Also *in vivo* PPS seems to be very effective similarly to the doses of other

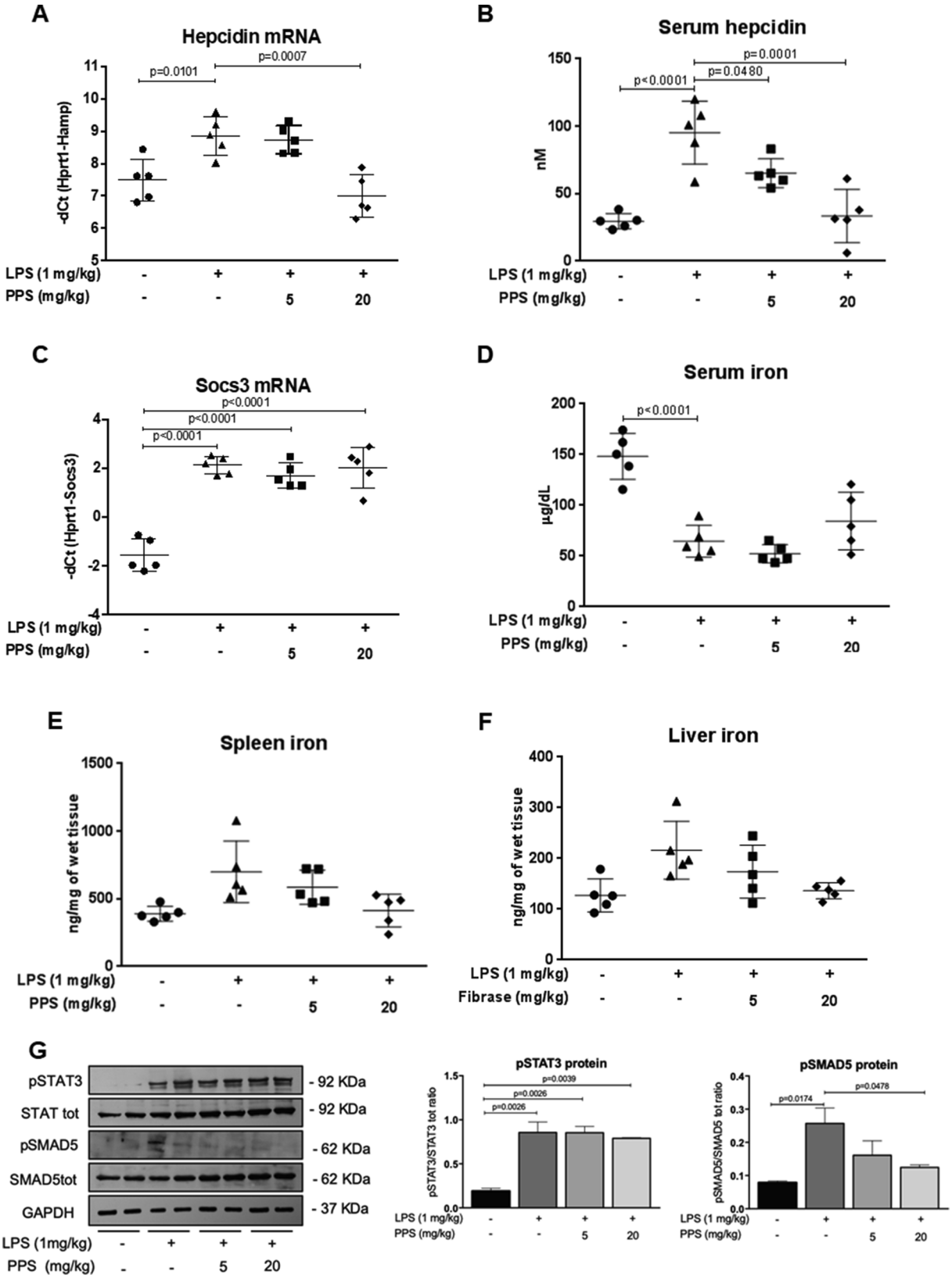

Fig. 7. A single treatment of PPS (20 mg/kg) abolished LPS-dependent induction of hepcidin in mice. Mice (8–9 weeks old) (5 mice per experimental group) were treated with LPS (1 mg/kg) with or without a single dose of PPS (20 mg/kg) and euthanized after 6 h. qRT-PCR was used for mRNA quantification of (A) hepcidin and (C) Socs3 in relationship to Hprt1 mRNA and expressed as -dCt (Hprt1-Hamp) or -dCt (Hprt1-Socs3). (B) Serum Hepcidin was measured by mass spectrometry. (D) Serum iron was measured by commercial kit. (E) Spleen and (F) Liver iron content was measured spectrophotometrically. (G) Western blotting analysis of pSTAT3, STAT3, pSMAD5, SMAD5tot and GAPDH (as calibrator of the samples). The graph showed the densitometry of pSTAT3/STAT3tot and pSMAD5/SMAD5tot comparing the untreated mice (-) to the treated ones (with LPS alone or in combination with PPS 5 or 20 mg/kg).

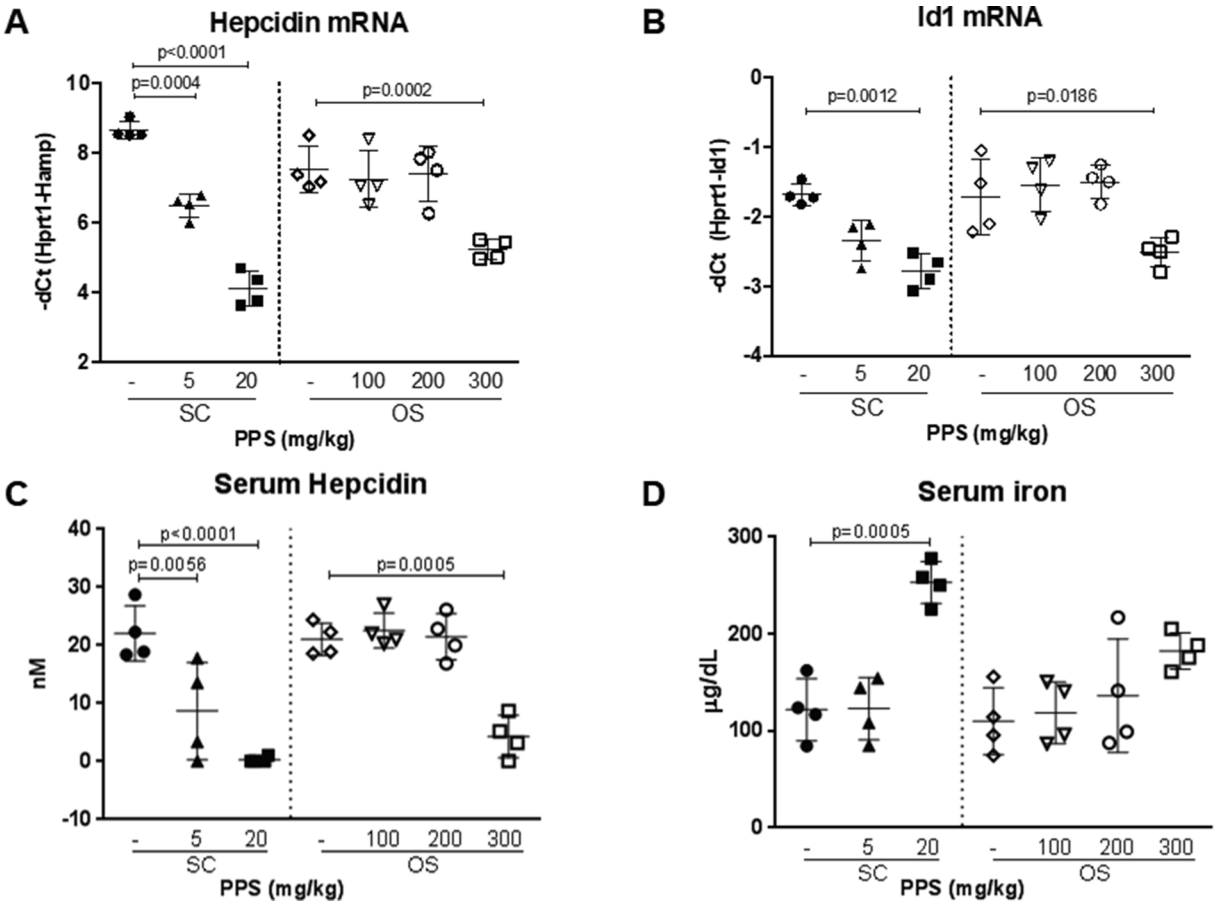

Fig. 8. Both subcutaneous and oral administration of PPS strongly inhibited liver hepcidin and increased serum iron in 6 h. Mice (8–9 weeks old) (4 mice per experimental group) were treated subcutaneously (SC) with PPS 5 and 20 mg/kg or PBS (as control) or orally by gavage (OS) with PPS (100–200–300 mg/kg) or PBS (as control) in single treatment and euthanized after 6 h. (A) Hepcidin and (B) Id1 mRNA was quantified by qRT-PCR in relationship to Hprt1 mRNA and expressed as -dCt (Hprt1-Hamp) or -dCt (Hprt1-Id1). (C) Serum hepcidin was measured by mass spectrometry. (C) Serum iron was measured spectrophotometrically by commercial kit.

heparin derivatives. The anti-hepcidin activity of PPS could be explained by the greater negative charge density distributed on the polysaccharide chain, that induce a high affinity for the basic clusters of the protein. Interestingly, we found that also oral PPS retains its anti-hepcidin activity, even if its absorption is low, about 6%. While the low oral bioavailability of heparins and LMW heparins is well known [27] in two patents was reported that oral administration di LMW-oversulfate heparin induce anticoagulant effects (*EP0496233 (A1)-1992-07-29-Pharmaceutical compositions for oral use containing low molecular weight heparin, their preparation and use. inventor Lattanzi Filippo, applicant Sclavo; EP1792621 (A1) Orally administrable heparin derivatives Inventor Torri G.; Naggi A. Richiedente: Debiopharm SA*).

A possible explanation of the oral bioavailability of both LMW-oversulfated heparin and PPS could be their ability to induce an increase of the intestinal epithelium permeability. Such interpretation is in agreement with the finding that the oral administration of dextransulfate, a highly negatively charged polysaccharide such as LMW-oversulfated heparin and PPS, is associated with the loss of the ZO-1 occluding in the tight junction complex (TJ) [28].

The effect on hepcidin expression and iron distribution is visible with 300 mg/kg PPS in single administration with a 50% reduction of hepcidin mRNA. Thus, the use of oral PPS to suppress hepcidin seems feasible, offering a promising strategy to complement the subcutaneous injection of PPS, especially in chronic treatment, but it requires further studies. For interstitial cystitis, the suggested oral treatment is 400 mg of PPS daily for a long period of time (186 months) which is well tolerated [28], thus it could be of interest, in the future, to try chronic treatments in mice with concentrations lower than those used in the acute treatments. Currently, the subcutaneous injection of PPS seems very effective in reducing hepcidin expression in terms of drug absorption, dosage (much lower than oral administration) and negligible side effects. A recent study of H.M. Suranji Wijekoon and colleagues showed that a subcutaneous chronic treatment for 30 days with 20 mg/kg/day PPS in rats with Rheumatoid Arthritis in collagen-induced arthritis model (CIA) showed promising anti-arthritic effects by ameliorating clinical and functional outcome without side effects [20], offering a good starting point to test PPS in chronic to control hepcidin.

In conclusion, in the present work we showed the possibility to use PPS as an efficient anti-hepcidin molecule, with marginal side effects. The PPS compound is already approved for the use in human and thus some steps of clinical trials were already performed and it could be advantageous in terms of timing and cost savings for the approval of PPS to control hepcidin *in vivo*.

CRediT authorship contribution statement

Michela Asperti: Investigation, Conceptualization, Writing - original draft. **Andrea Denardo:** Investigation. **Magdalena Gryzik:** Investigation. **Annalisa Castagna:** Investigation. **Domenico Girelli:** Writing - review & editing. **Annamaria Naggi:** Writing - review & editing. **Paolo Arosio:** Writing - review & editing. **Maura Poli:** Conceptualization, Supervision, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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