Comparative analysis of oral and intravenous iron therapy in rat models of inflammatory anemia and iron deficiency

Lara Valente de Souza,[1,2] Alexander Hoffmann,[1,2] Christine Fischer,[1] Verena Petzer,[1] Malte Asshoff,[1] Igor Theurl,[1] Piotr Tymoszuk,[1] Markus Seifert,[1,2] Natascha Brigo,[1] Richard Hilbe,[1] Egon Demetz,[1] Laura Von Raffay,[1] Sylvia Berger,[1] Marina Barros-Pinkelnig[1] and Guenter Weiss[1,2]

[1]Department of Internal Medicine II, Infectious Diseases, Immunology, Rheumatology and [2]Christian Doppler Laboratory for Iron Metabolism and Anemia Research, Medical University of Innsbruck, Innsbruck, Austria.

Correspondence: G. Weiss
guenter.weiss@i-med.ac.at

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Abstract

Anemia is a major health issue and associated with increased morbidity. Iron deficiency anemia (IDA) is the most prevalent, followed by anemia of chronic disease (ACD). IDA and ACD often co-exist, challenging diagnosis and treatment. While iron supplementation is the first-line therapy for IDA, its optimal route of administration and the efficacy of different repletion strategies in ACD are elusive. Female Lewis rats were injected with group A streptococcal peptidoglycan-polysaccharide (PG-APS) to induce inflammatory arthritis with associated ACD and/or repeatedly phlebotomized and fed with a low iron diet to induce IDA, or a combination thereof (ACD/IDA). Iron was either supplemented by daily oral gavage of ferric maltol or by weekly intravenous (i.v.) injection of ferric carboxymaltose for up to 4 weeks. While both strategies reversed IDA, they remained ineffective to improve hemoglobin (Hb) levels in ACD, although oral iron showed slight amelioration of various erythropoiesis-associated parameters. In contrast, both iron treatments significantly increased Hb in ACD/IDA. In ACD and ACD/IDA animals, i.v. iron administration resulted in iron trapping in liver and splenic macrophages, induction of ferritin expression and increased circulating levels of the iron hormone hepcidin and the inflammatory cytokine interleukin-6, while oral iron supplementation reduced interleukin-6 levels. Thus, oral and i.v. iron resulted in divergent effects on systemic and tissue iron homeostasis and inflammation. Our results indicate that both iron supplements improve Hb in ACD/IDA, but are ineffective in ACD with pronounced inflammation, and that under the latter condition, i.v. iron is trapped in macrophages and may enhance inflammation.

Introduction

Anemia is a major health issue and its prevalence reaches 30% of the world's population.[1,2] Iron deficiency anemia (IDA) is the most prevalent form,[3] followed by anemia of chronic disease (ACD), also known as anemia of inflammation (AI).[4,5] In many cases, ACD can be associated with IDA, mostly due to concomitant blood loss in association with chronic inflammatory disorders.[2,4,6,7]

Iron is necessary for numerous cellular functions and its reduced availability can lead to impaired metabolic activity and mitochondrial dysfunction. Opposing, iron overload can cause cellular malfunction by catalyzing toxic radical formation. In particular, iron is crucial for effective erythropoiesis, as 25-30 mg is needed daily mainly for heme biosynthesis to generate erythrocytes.[8-10] Approximately 90% of iron used for this process originates from macrophages, responsible for engulfing senescent red blood cells (RBC) and redistributing the iron to the bone marrow (BM), whereas only 5-10% of the daily needs from iron are covered by dietary iron absorption in the duodenum in humans,[2,11,12] while this number can reach 25% in healthy rats.[13]

Systemic iron homeostasis is controlled by the hepatocyte-derived peptide hepcidin (encoded by the gene *Hamp*). It binds to ferroportin (encoded by the gene *Fpn1*), the only known cellular iron exporter, leading to its lysosomal internalization and degradation.[14] High plasma iron concentration but also inflammatory stimuli induce hepcidin production and diminishes Fpn1 expression, thereby

resulting in macrophage iron retention and reduced duodenal iron absorption.[2-4] Hallmarks are low circulating iron and increased ferritin (FT) levels.[15] Over time, inflammation-driven iron retention results in the development of ACD. In addition, negative effects of cytokines on erythroid progenitor cell differentiation, on the biological activity of the red cell hormone erythropoietin (EPO) and on the circulatory half-life of erythrocyte further contribute to ACD development.[4,16] In contrast, low iron availability inhibits hepcidin production and enables efficient dietary iron absorption and iron redistribution from macrophages.[2,8,15]

IDA develops due to absolute iron deficiency, resulting from blood losses and/or inadequate iron absorption.[3,17] ACD is characterized as normochromic/normocytic, whereas IDA presents with a microcytic/hypochromic phenotype along with low circulating iron levels, reduced saturation of iron with transferrin (TSAT) and reduced FT.[17-19] While IDA is the consequence of absolute or true iron deficiency, inflammation-driven iron retention in macrophages causes functional iron deficiency, making the metal unavailable for erythropoietic progenitors. Thus, IDA and ACD have different pathophysiology and diagnostic hallmarks.[2-4,18,20,21]

A specific clinical challenge is to differentiate patients with ACD from those with ACD and combined true iron deficiency (ACD/IDA). This is of importance because these two specific groups of patients may need different therapies.[3,4,18] Although no single laboratory marker accurately provides a correct differential diagnosis, subjects with ACD/IDA have lower circulating hepcidin levels, lower hemoglobin (Hb) reticulocyte content, a higher percentage of hypochromic RBC or an increased FT index as compared to ACD individuals.[18,19,21-23]

The best approach towards anemia is to identify and treat its underlying cause. While in IDA iron supplementation is the treatment of choice to replenish depleted iron stores,[17,18,24] the efficacy of this measure for the treatment of ACD is less clear. It may largely depend on the underlying disease and its inflammatory activity, the mode of iron administration and its potential off-target effects, especially considering that ACD may result from a defense strategy of the body to limit the availability of the nutrient iron to invading microbes or malignant cells.[4,17,24-26]

Iron can be therapeutically administrated either in oral or intravenous (i.v.) forms. In most cases of iron deficiency, oral iron supplementation is the first choice. However, gastrointestinal side effects may limit therapeutic adherence and inflammation-driven hepcidin levels reduce duodenum iron absorption.[2,4,15,27,28] In such situations, i.v. iron is the preferred treatment. Specifically, new carbohydrate preparations enable higher single dose applications.[7,17,18,24,29] However, iron carbohydrate complexes are primarily ingested by macrophages and the release of iron into the circulation via FPN1 is controlled by hepcidin,[14,15,30] thereby trapping iron in macrophages in situations when hepcidin levels are high.

In order to systemically study the pharmacokinetics and therapeutic efficacy of iron supplementation in IDA, ACD and ACD/IDA, we performed a comparative analysis of oral iron therapy with ferric maltol (F. Maltol) and i.v. iron treatment with ferric carboxymaltose (FCM), employing well established rat models.[15,31,32]

Methods

Animals

The animal experiments were approved by the Medical University of Innsbruck and the Austrian Federal Ministry of Science and Research (BMBWF-66.011/0138-WF/V/3b/2016). We used a widely used model of IDA, ACD and a combination thereof employing 6-week-old female Lewis rats as previously described.[15] For iron treatments we used either F. Maltol for oral iron supplementation or FCM for i.v. iron therapy, two clinically established drugs for therapy of iron deficiency. Further details are provided in the *Online Supplementary Appendix*.

Blood sample analysis and iron measurements, evaluation of tissue iron homeostasis and erythropoiesis

Analysis of blood parameters, tissue iron homeostasis, and determination of erythropoietic activity was performed as previously described and further information is given in the *Online Supplementary Appendix*.[32]

Statistical analysis

Statistical analysis was carried out using GraphPad Prism version 8 for Windows (GraphPad Software). Data are presented as mean ± standard deviation (SD) unless otherwise specified. Significant differences between groups are specified in each figure legend and were determined using either a one way ANOVA with Dunnet's multiple comparisons test, or two way ANOVA with Sidak's multiple comparisons test. The sample size for each animal experiment was estimated based on previous experience, usually with five animals per control group and five to eight animals per untreated and treatment groups. According to each experiment, rats were randomly assigned to the different groups. A P value <0.05 was used as the significance threshold.

Results

Effects of oral and intravenous iron on systemic and tissue iron homeostasis, erythropoiesis and hemoglobin levels in iron deficiency anemia

We first investigated the therapeutic effects of iron supplementation in the IDA model. Untreated IDA rats had the

major characteristics of IDA caused by low iron intake and blood loss, with microcytic and hypochromic anemia, low plasma iron and increased reticulocyte counts (Figure 1A to E), as compared to control animals. Supplementation of either oral or i.v. iron at both dosages had a comparable efficacy in ameliorating anemia at the end of the treatment period resulting in normalization of Hb levels, red cell indices and plasma iron levels. In contrast, reticulocyte counts were reduced as compared to untreated IDA animals, which is in line with therapeutic correction of anemia (Figure 1A to E). When evaluating erythroid progenitor subpopulations in the BM, we found that the increase of pro-erythroblasts in IDA rats was reversed by iron treatment, while orthochromatic erythroblasts were significantly higher in IDA and iron treated animals than in controls (Figure 1F).

We then investigated iron homeostasis in the liver, duodenum and spleen. As expected, *Hamp* mRNA levels were significantly reduced in untreated IDA rats compared to controls and increased upon iron supplementation (Figure 2A). Oral iron treatment resulted in significantly lower *Hamp* mRNA levels than in control animals. Intravenously iron-supplemented animals, specifically those which received the higher dosage, presented with the highest *Hamp* expression, which even exceeded that of control animals (Figure 2A). The mRNA expression of the hepcidin modulator bone morphogenetic protein 6 (*Bmp6*)[33] was reduced in IDA as compared to controls but increased with iron supplementation (Figure 7A). Similar trends were seen when measuring hepcidin levels in plasma, with significant differences between control and rats treated with i.v. iron (Figure 2B). In parallel, plasma EPO levels were higher in IDA untreated animals and returned to control levels with iron supplementation (Figure 7B). While a reduction of liver iron content was evident in untreated IDA compared to control rats and iron levels returned to the value of controls in orally iron supplemented rats, we found a dose-dependent increase in liver iron concentra-

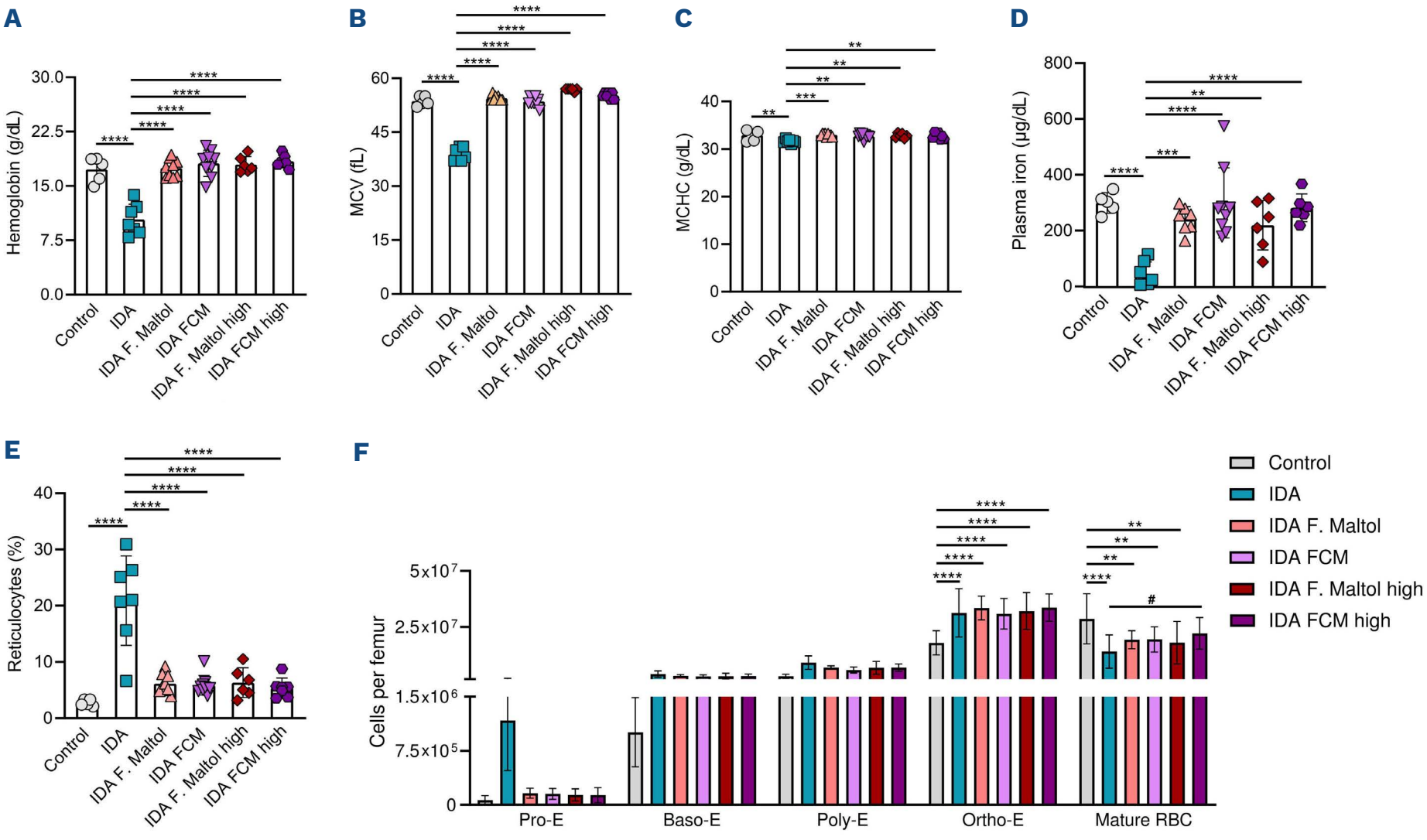

Figure 1. Oral and intravenous iron supplementations improve blood count parameters and bone marrow erythropoiesis in a rat model of iron deficiency anemia. (A) Hemoglobin (Hb) levels. (B) Mean corpuscular volume (MCV). (C) Mean corpuscular Hb concentration (MCHC). (D) Plasma iron measurement. (E) Reticulocytes were analyzed by flow cytometry. (F) Bone marrow populations consisting of: pro-erythroblasts (Pro-E; population I), basophilic erythroblasts (Baso-E; population II), polychromatic erythroblasts (Poly-E; population III), orthochromatic erythroblasts (Ortho-E; population IV) and mature red blood cells (RBC) (Mature RBC; population V) were analyzed by flow cytometry. One-way ANOVA with Dunnett's multiple comparisons test between iron deficiency anemia (IDA) and all other groups was applied for panels (A to E). Two-way ANOVA with Dunnett's multiple comparisons test for each population, between control and IDA groups with all groups was applied for panel (F). **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. #*P*<0.05. Results are shown as mean ± standard deviation. Results are from one representative experiment with 5-8 animals per group. F Maltol: ferric maltol; FCM: ferric carboxymaltose.

tions following i.v. iron supplementation (Figure 2C).

When studying the expression of essential iron metabolism genes in the liver, we found that *Fpn1* mRNA levels were lower in IDA than in control animals, but slightly increased with iron supplementation (*Online Supplementary Figure S4C*). Of note, while FPN1 protein levels were low in untreated IDA and control rats and did not increase with oral iron treatment, an induction was observed with i.v. iron treatment (Figure 2C; *Online Supplementary Figure S4F*). A similar effect was observed for the iron storage protein FT, which increased with iron supplementation, most prominently with i.v. iron treatment (Figure 2C; *Online Supplementary Figure S4G*). These alterations of FPN1 and FT expression were in good association with respective changes in liver iron content (Figure 2C). The expression of nuclear receptor co-activator 4 (NCOA4)

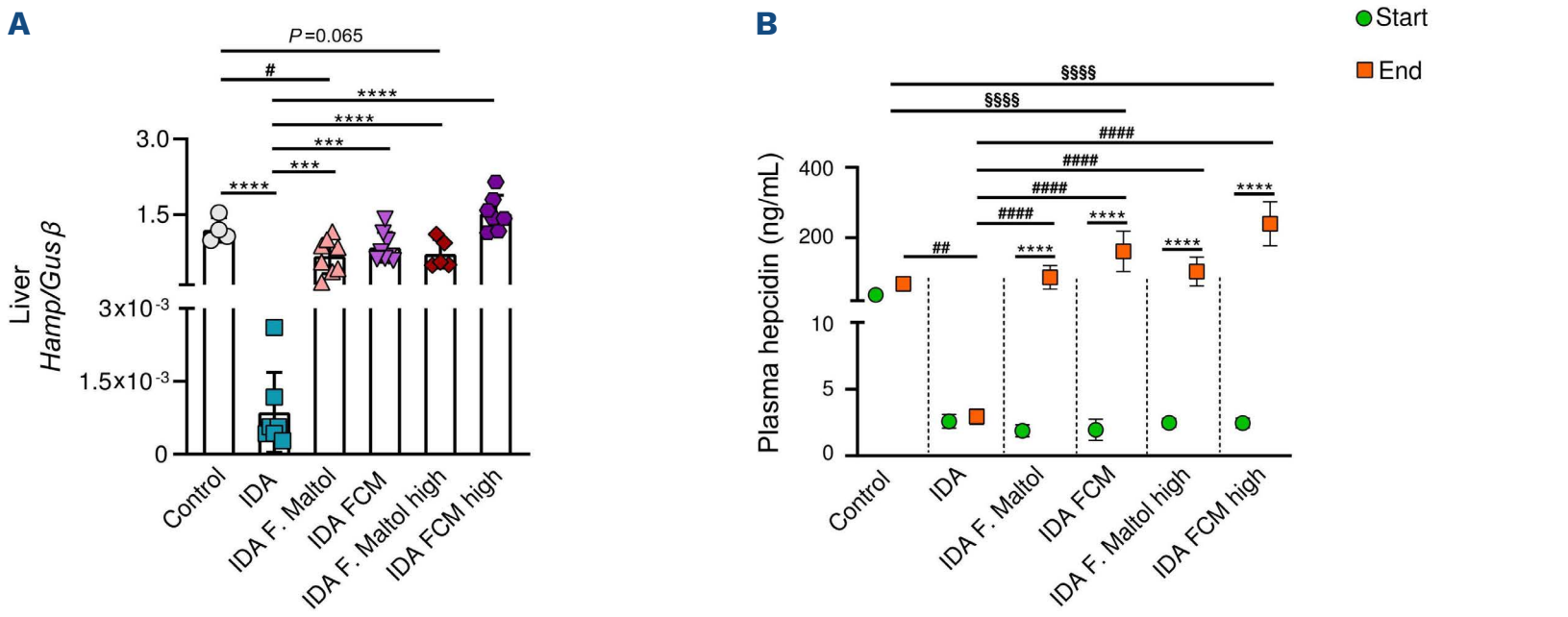

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Figure 2. Oral and more prominently intravenous iron supplementations increase hepcidin levels in the liver and iron concentration in the liver and spleen in a rat model of iron deficiency anemia. (A) Liver *Hamp* mRNA levels. (B) Hepcidin plasma levels at baseline and end of the observation period measured by enzyme-linked immunosorbent assay. (C) Liver iron concentration and protein expression measured by western blot (WB) of TFR1, NCOA4, FPN1 and FT; β-actin (ACTB) was used as the housekeeping protein. (D) Spleen iron concentration and protein expression measured by WB of TFR1, NCOA4, FPN1 and FT; β-actin (ACTB) was used as the housekeeping protein. One-way ANOVA with Dunnett´s multiple comparisons test between control and iron deficiency anemia (IDA) against all other groups was applied for panel (A), and IDA against all other groups for panels (C and D). For results shown in (B), two-way ANOVA with Sidak´s multiple comparisons test was used for analyzing differences between baseline and end of the observation period of each group. **P*<0.05, ***P*<0.01, ****P*<0,001, *****P*<0.0001. The same test was used to analyze differences at end of the experiment between control and IDA groups with all other groups. $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0,001, $^{\#\#\#\#}P$<0.0001. $^{\$\$\$\$}P$<0.0001. Results are shown as mean ± standard deviation. Results are from 1 representative experiment with 5-8 animals per group. F Maltol: ferric maltol ; FCM: ferric carboxymaltose.

(encoded by the gene *Ncoa4*), which is the cargo receptor for the degradation of FT and re-utilization of stored iron in cells[34] was insignificantly altered between the different groups (Figure 2C; *Online Supplementary Figure S4B* and *E*). Finally, transferrin receptor protein 1 (TFR1) (encoded by the gene *Tfr1*) mRNA and protein levels were significantly increased in the IDA group and reduced by iron supplementation, being in line with the known regulation of this gene by iron deficiency and iron regulatory proteins (Figure 2C; *Online Supplementary Figure S4A* and *D)*.[8]

We also measured the mRNA expression of the divalent metal transporter-1 (encoded by the gene Dmt1) in the duodenum, because it is the major iron transporter shuttling dietary and orally supplemented iron from the intestinal lumen into the enterocyte.[35,36] Duodenal *Dmt1* mRNA expression increased in untreated IDA as compared to other groups, and iron supplementation reduced *Dmt1* mRNA levels to that found in controls (*Online Supplementary Figure S3A*).

We further analyzed the effects of IDA and iron supplementation in the spleen, which largely consists of macrophages involved in iron recycling and delivery for erythropoiesis.[12] Accordingly, splenic iron concentration was reduced in IDA compared to controls but remained unchanged with oral iron supplementation (Figure 2D). Meanwhile, i.v. iron therapy resulted in a significant increase in splenic iron content being compatible with acquisition and storage of iron-carbohydrate complexes within macrophages.[2] This was paralleled by concomitant alterations of FT and FPN1 protein expression in IDA with and without different iron treatments, while *Fpn1* mRNA levels did not change with iron supplementation (Figure 2D; *Online Supplementary Figure S4J*, *M* and *N*). NCOA4 protein levels and TFR1 protein and mRNA levels increased with IDA, both indicative of increased iron needs, and returned to control levels with iron treatments (Figure 2D; *Online Supplementary Figure S4H, I, K* and *L*).

Taken together, these results show that in the setting of IDA, oral and i.v. iron were equally effective in correcting anemia, but surplus i.v. iron was stored in the liver and spleen, presumably within macrophages.

Effects of oral and intravenous iron treatment on systemic and tissue iron homeostasis, erythropoiesis and hemoglobin levels in anemia of chronic disease

At the end of the treatment period, neither oral nor i.v. iron significantly changed Hb levels, MCV or MCHC as compared to untreated ACD animals (Figure 3A to C). Plasma iron levels were reduced in ACD and remained unchanged following i.v. iron treatment, whereas oral iron therapy resulted in a slight but insignificant increase (Figure 3D). Of interest, oral iron supplementation approximated the number of reticulocytes to the control group, while for intravenously treated animals the reticulocyte counts were higher and comparable to untreated ACD animals (Figure 3E). In the BM, ACD rats and intravenously but not orally iron-supplemented animals had significantly lower number of orthochromatic erythroblasts than control animals (Figure 3F).

We found that, at the end of the study period, *Hamp* mRNA expression in the liver was not significantly different between control and untreated ACD animals. Oral iron treatment had no effect on *Hamp* levels, whereas i.v. iron therapy resulted in a significant increase in *Hamp* expression compared to the ACD group (Figure 4A). These changes were mirrored by hepcidin levels in plasma, which significantly increased with i.v. iron therapy over time (Figure 4B), being in line with higher liver iron content following i.v. therapy of ACD animals (Figure 4D). Interestingly, mRNA *Bmp6* expression was lower in the ACD-untreated animals than in the control groups and no difference was seen with iron supplementation (Figure 7C). Plasma EPO levels were higher in untreated and intravenously supplemented ACD compared to control animals, while reduced in the groups receiving oral iron (Figure 7D). Further, we also analyzed for changes in IL-6 levels, a marker of inflammation and major inducer of hepcidin expression.[2] While IL-6 levels were increased but not different between the treatment groups at study initiation, oral iron therapy significantly reduced plasma IL-6 levels. Meanwhile, they remained unchanged in untreated ACD rats over time, whereas they significantly increased in animals receiving higher i.v. iron (Figure 4C).

In the liver, we did not observe obvious changes in the

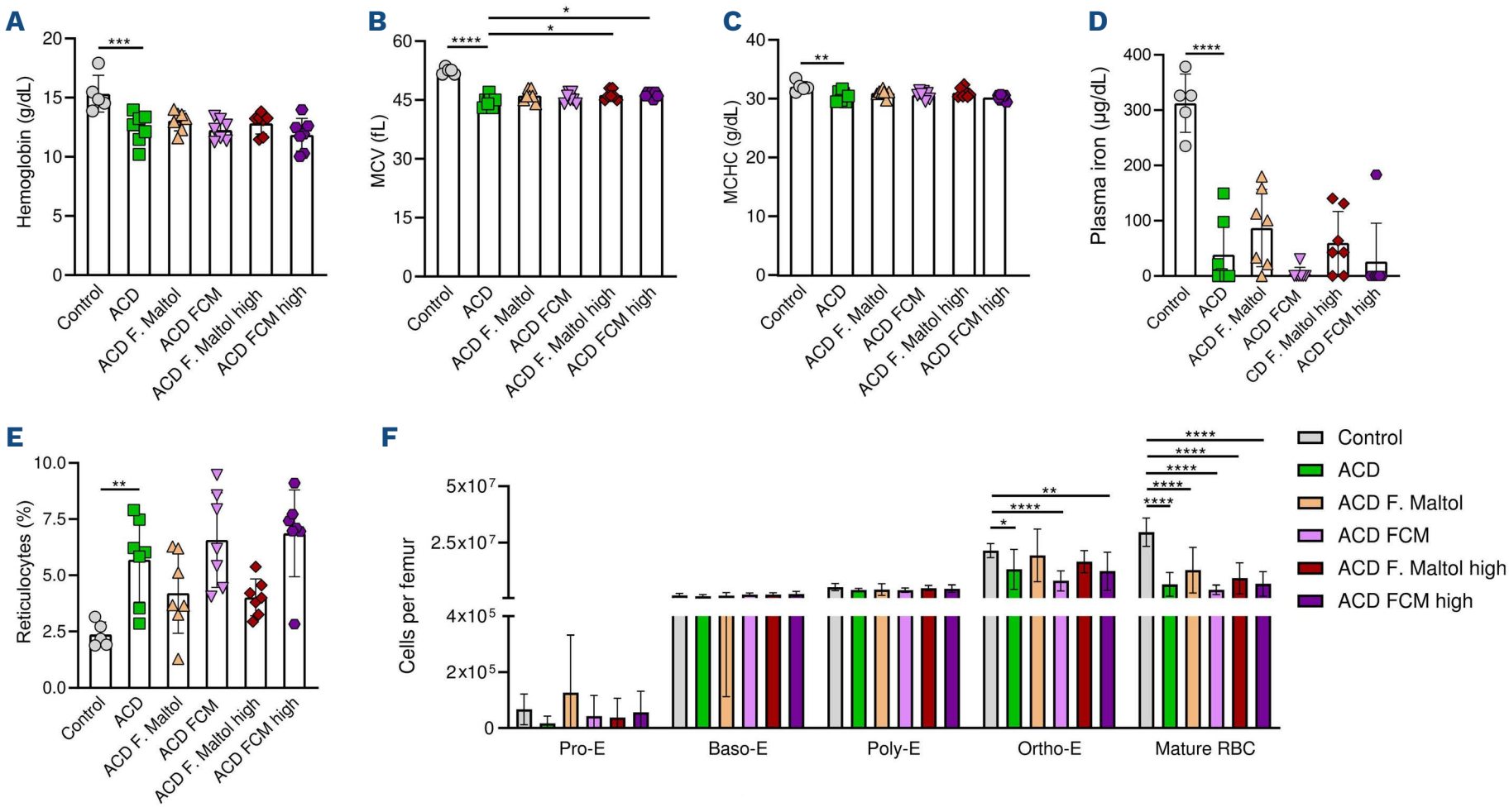

Figure 3. Oral and especially intravenous iron supplementation do not ameliorate blood count parameters and bone marrow erythropoiesis in a rat model of anemia of chronic disease. (A) Hemoglobin (Hb) levels. (B) Mean corpuscular volume (MCV). (C) Mean corpuscular Hb concentration (MCHC). (D) Plasma iron concentration. (E) Reticulocytes were analyzed by flow cytometry. (F) Bone marrow populations consisting of proerythroblasts (Pro-E; population I), basophilic erythroblasts (Baso-E; population II), polychromatic erythroblasts (Poly-E; population III), orthochromatic erythroblasts (Ortho-E; population IV) and mature red blood cells (RBC) (Mature RBC; population V) were analyzed by flow cytometry. One-way ANOVA with Dunnett's multiple comparisons test between anemia of chronic disease (ACD) and all other groups was applied for panels (A to E). Two-way ANOVA with Dunnett's multiple comparisons test for each population, between control and all groups was applied for panel (F). **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. Results are shown as mean ± standard deviation. Results are from 1 representative experiment with 5-8 animals per group. F Maltol: ferric maltol ; FCM: ferric carboxymaltose.

mRNA expression of *Fpn1, Tfr1* or *Ncoa4* (*Online Supplementary Figure S5A* to *C*). Despite high hepcidin levels, FPN1 protein levels (Figure 4D; *Online Supplementary Figure S5F*) did not change with i.v. iron treatment, and TFR1 protein concentrations were higher in untreated ACD than in control animals as previously described[15] (Figure 4D; *Online Supplementary Figure S5D*). NCOA4 protein expression showed no consistent regulation, whereas FT levels were higher with i.v. iron treatment than in untreated and orally iron supplemented ACD rats (Figure 4D; *Online Supplementary Figure S5E* and *G*). *Dmt1* mRNA expression in the duodenum did not differ between the groups (*Online Supplementary Figure S3B*).

A similar trend was observed in the spleen, where i.v. iron treatment caused increased splenic iron concentrations and higher FT levels compared to all other groups (Figure 4E; *Online Supplementary Figure S5N*). In agreement with the effects of cytokines and hepcidin on FPN1 expression,[14,37] we observed its reduction in untreated ACD compared to control rats (Figure 4E; *Online Supplementary Figure S5J* and *M*). Iron supplementation increased FPN1 protein expression, which was most prominent with high i.v. iron treatment, that also resulted in reduced protein expression of the FT decay receptor NCOA4 (Figure 4E; *Online Supplementary Figure S5J, L* and *M*). Finally, *Tfr1* mRNA and protein levels were higher in the ACD group and i.v. iron-treated animals than in controls or rats treated with oral iron therapy (Figure 4E; *Online Supplementary Figure S5H* and *K*).

These data indicate that with advanced inflammation, both oral and i.v. iron cannot improve Hb levels. However, we observed striking differences of oral *versus* i.v. iron therapy on systemic and tissue iron homeostasis.

Effects of oral and intravenous iron on systemic and tissue iron homeostasis, erythropoiesis, and hemoglobin levels in anemia of chronic disease/iron deficiency anemia

We then investigated the effects of iron supplementation in ACD rats with combined true iron deficiency (ACD/IDA). Compared to ACD, ACD/IDA rats had lower Hb, MCV, MCHC and plasma iron levels (Figure 5A to D), while reticulocyte

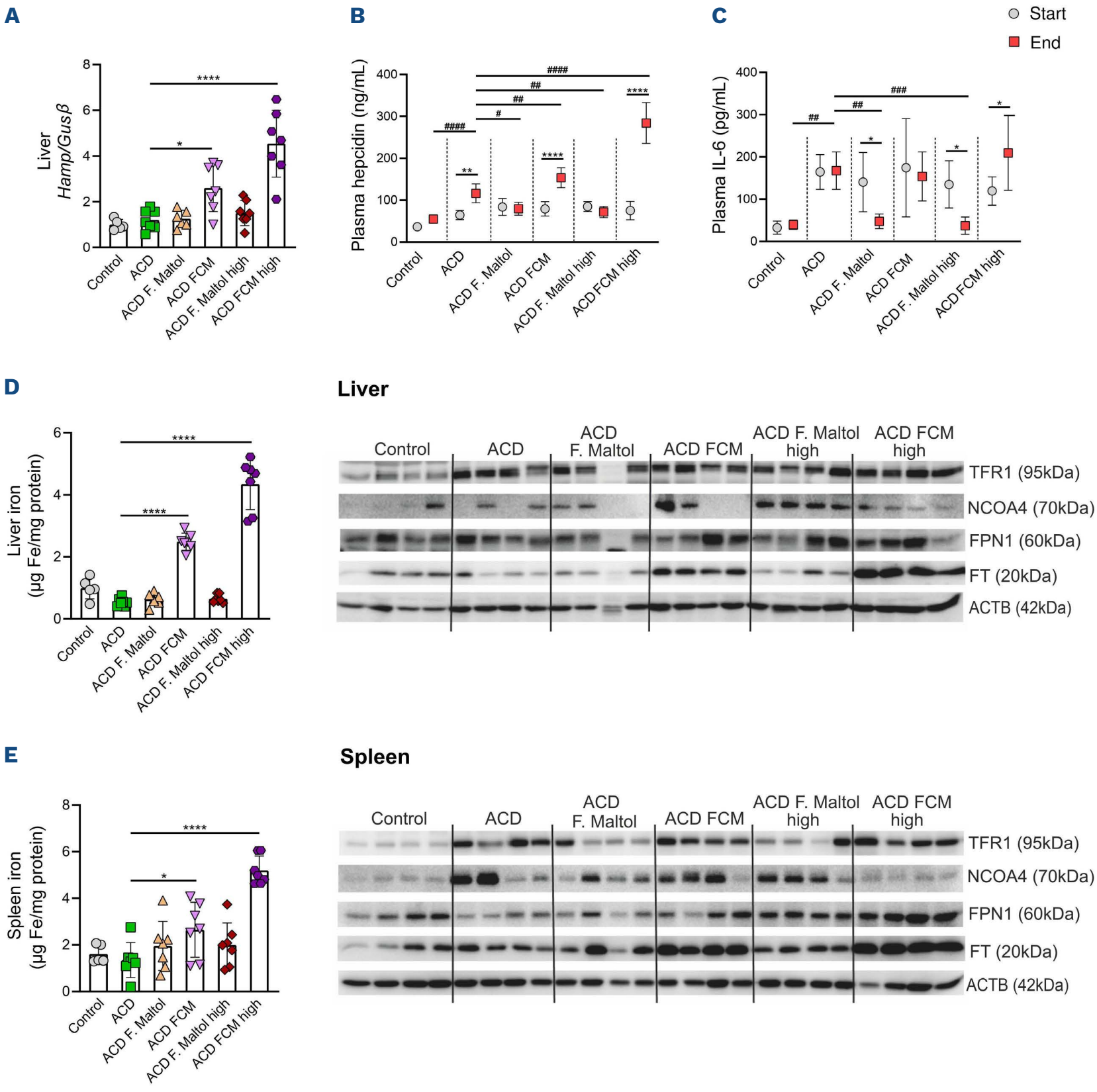

Figure 4. Intravenous but not oral iron supplementation increases inflammatory parameters in the liver, as well as iron concentration in the liver and spleen in a rat model of anemia of chronic disease. (A) *Hamp* mRNA levels. (B) Plasma hepcidin and (C) interleukin-6 (IL-6) (values for healthy animals ranged from 15,4 pg/mL to 57,6 pg/mL) levels at baseline and end of the observation period measured by enzyme-linked immunosorbent assay. (D) Liver iron concentration and protein expression measured by western blot (WB) of TFR1, NCOA4, FPN1 and FT; β-actin (ACTB) was used as the housekeeping protein. (I) Spleen iron concentration and protein expression measured by WB of TFR1, NCOA4, FPN1 and FT; β-actin (ACTB) was used as the housekeeping protein. One-way ANOVA with Dunnett´s multiple comparisons test between ACD and all other groups was applied for results shown in (A, D and E). For results shown in (B and C), two-way ANOVA with Sidak´s multiple comparisons test was used for analyzing differences between baseline and end of the observation period of each group. **P*<0.05, ***P*<0.01, ****P*<0,001, *****P*<0.0001. Two-way ANOVA with Sidak´s multiple comparisons test was used to analyze differences between ACD with all other groups at end of the experiment. #*P*<0.05, ##*P*<0.01, ###*P*<0,001, ####*P*<0.0001. Results are shown as mean ± standard deviation. Results are from one representative experiment with 5-8 animals per group. F Maltol: ferric maltol ; FCM: ferric carboxymaltose.

counts were higher in the latter (Figure 5E). Animals that received oral and i.v. iron supplementation significantly improved Hb concentrations and MCV, but not MCHC, compared to untreated ACD/IDA animals (Figure 5A to C), while reticulocyte numbers were not different from untreated ACD/IDA animals (Figure 5E). When analyzing the BM (Figure 5F), untreated ACD/IDA had higher amounts of pro-erythroblasts than all other groups, which were reduced with both iron treatments. Meanwhile, orthochromatic erythroblasts were higher in ACD/IDA and all

treatment groups than in ACD, except for the group supplemented with high i.v. iron. Numbers of mature RBC were lower in ACD and ACD/IDA compared to the controls and not significantly changed by iron treatment (Figure 5F).

When analyzing for changes in iron homeostasis, we found that ACD/IDA animals had significantly lower *Hamp* mRNA expression and plasma hepcidin levels than control and ACD rats (Figure 6A and B), reconfirming published evidence.[15,38] Hepatic *Hamp* mRNA expression was higher in the ACD group and in ACD/IDA iron-supplemented animals (Figure 6A). Plasma hepcidin concentrations (Figure 6B) significantly increased in groups receiving i.v. iron, but not in orally iron-treated rats, when compared to baseline levels. This is paralleled by appropriate changes in liver iron concentrations (Figure 6D), being also significantly increased with i.v. iron therapy (Figure 6B and D). Of note, we found no changes in hepatic *Bmp6* mRNA expression between the groups (Figure 7E). EPO plasma levels were higher in ACD/IDA untreated animals compared to control and ACD, and they decreased more significantly with oral than with i.v. iron treatment (Figure 7F). IL-6 plasma concentrations were not different between ACD and ACD/IDA animals. Of interest, compared to baseline values, IL-6 levels decreased with oral iron treatment, whereas they increased following i.v. iron therapy (Figure 6C).

When studying iron metabolism in the liver, we found that FT protein expression and FPN1 protein and mRNA levels increased with i.v. iron treatment (Figure 6D; *Online Supplementary Figure S6C, F* and *G*). NCOA4 protein levels and mRNA were reduced in all anemia and treatment groups compared to controls (Figure 6D; *Online Supplementary Figure S6B* and *E*).

Duodenal *Dmt1* mRNA expression was significantly increased in ACD/IDA as compared to ACD and controls, but then significantly reduced by either form of iron therapy

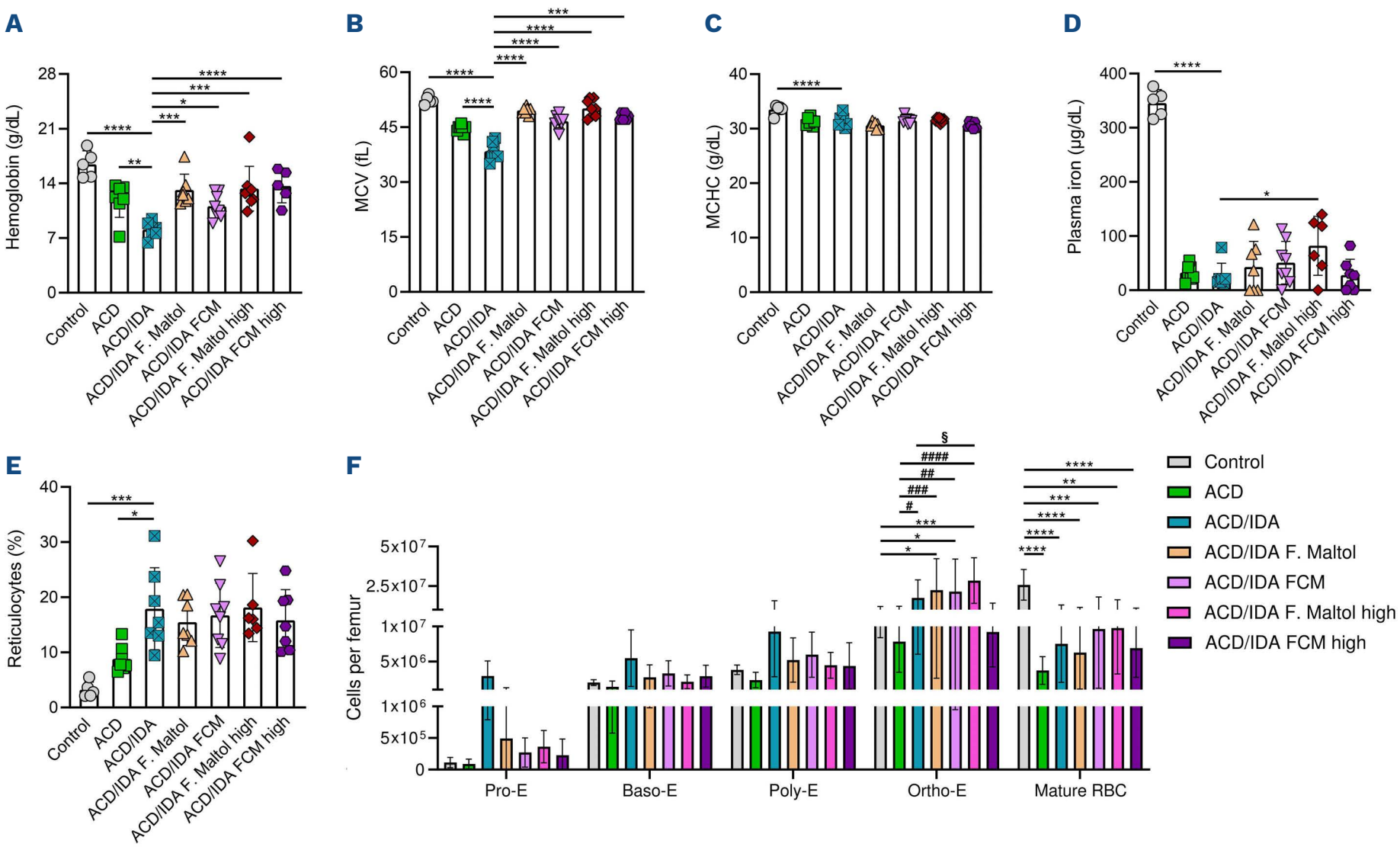

Figure 5. Oral and intravenous iron supplementation improve blood count parameters and bone marrow erythropoiesis in a rat model of anemia of chronic disease combined with iron deficiency anemia. (A) Hemoglobin (Hb) levels. (B) Mean corpuscular volume (MCV). (C) Mean corpuscular Hb concentration (MCHC). (D) Plasma iron concentration. (E) Reticulocytes were analyzed by flow cytometry. (F) Bone marrow populations consisting of pro-erythroblasts (Pro-E; population I), basophilic erythroblasts (Baso-E; population II), polychromatic erythroblasts (Poly-E; population III), orthochromatic erythroblasts (Ortho-E; population IV) and mature red blood cells (RBC) (Mature RBC; population V) were analyzed by flow cytometry. One-way ANOVA with Dunnett's multiple comparisons test between anemia of chronic disease combined with iron deficiency anemia (ACD/IDA) and all other groups was applied for panels (A to E). Two-way ANOVA with Dunnett's multiple comparisons test for each population, between control, ACD and ACD/IDA with all groups was applied for panel (F). **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001, $^{\#}$*P*<0.05, $^{\#\#}$*P*<0.01, $^{\#\#\#}$*P*<0.001, $^{\#\#\#\#}$*P*<0.0001, §*P*<0.05. Results are shown as mean ± standard deviation. Results are from one representative experiment with 5-8 animals per group. F Maltol: ferric maltol ; FCM: ferric carboxymaltose

(*Online Supplementary Figure S3C*).

In the spleen, ACD/IDA rats presented with lower iron levels than untreated ACD animals, and iron supplementation increased iron contents, again most pronounced with high i.v. iron (Figure 6E). FPN1 protein expression was slightly lower in ACD and ACD/IDA when compared to controls, but not significantly altered with iron treatment (Figure 6E; *Online Supplementary Figure S6M*). Iron stores, as reflected by FT protein levels, increased with iron treatment, being in line with data from determination of spleen iron content (Figure 6E; *Online Supplementary Figure S6N*). Taken together, these results show that in rats with ACD/IDA, both iron sources are capable of ameliorating anemia, but i.v. iron induces systemic hepcidin expression and IL-6 formation, along with iron deposition in the liver and spleen.

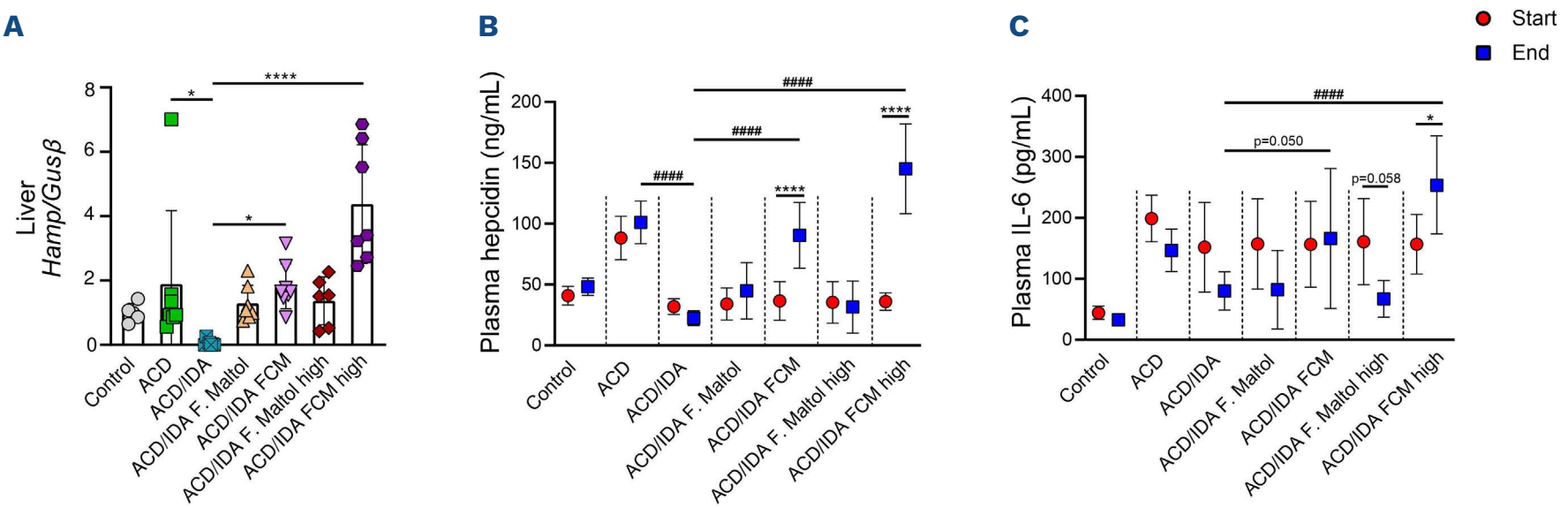

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Figure 6. Intravenous iron supplementation increases inflammatory parameters and iron concentration in the liver, as well as iron concentration in the spleen, while oral iron supplementation decreases interleukin-6 levels in a rat model of anemia of chronic disease combined with iron deficiency anemia. (A) *Hamp* mRNA levels. (B) Plasma hepcidin and (C) interleukin-6 (IL-6) (values for healthy animals ranged from 15,4 pg/mL to 57,6 pg/mL) levels at baseline and end of the observation period measured by enzyme-linked immunosorbent assay. (D) Liver iron concentration and protein expression measured by western blot (WB) of TFR1, NCOA4, FPN1 and FT; β-actin (ACTB) was used as the housekeeping protein. (I) Spleen iron concentration and protein expression measured by WB of TFR1, NCOA4, FPN1 and FT; β-actin (ACTB) was used as the housekeeping protein. One-way ANOVA with Dunnett´s multiple comparisons test between anemia of chronic disease combined with iron deficiency anemia (ACD/IDA) and all other groups was applied for results shown in panels (A, D and E). For results shown in panels (B and C), two-way ANOVA with Sidak´s multiple comparisons test was used for analyzing differences between baseline and end of the observation period of each group. **P*<0.05, ***P*<0.01, ****P*<0,001, *****P*<0.0001. The same test was used to analyze differences between ACD/IDA with other groups at end of the experiment. ####*P*<0.0001. Results are shown as mean ± standard deviation. Results are from 1 representative experiment with 5-8 animals per group.

Iron distribution in the liver and spleen differs depending on iron source and dose

We then analyzed for iron distribution in the liver (Figure 8; *Online Supplementary Figure S8A* to *C*) and spleen (*Online Supplementary Figure S9*) with Prussian blue staining. Further, we performed immunofluorescence in the liver to identify resident macrophages, known as Kupffer cells (*Online Supplementary Figure S7*).

The liver and spleen of untreated IDA and ACD/IDA groups were iron deficient, while untreated ACD controls showed iron retention within Kupffer cells in the liver and white pulp of the spleen, being compatible with macrophage iron accumulation due to inflammation (Figure 8; *Online Supplementary Figure S9*).

Oral iron supplementation in IDA animals results in the appearance of iron in hepatocytes, specifically in the F. Maltol high group. Intravenous iron therapy resulted in iron accumulation in Kupffer cells, compatible with uptake of iron-carbohydrate complexes by macrophages. Of interest, iron was also found in hepatocytes with high dose i.v. therapy (Figure 8). In the spleen, i.v. administration led to iron accumulation in white and red pulp, while groups receiving F. Maltol had similar iron distribution to untreated animals (*Online Supplementary Figure S9*).

Orally iron-supplemented ACD groups presented with liver and spleen iron distribution comparable to untreated ACD rats (Figure 8; *Online Supplementary Figure S9*). ACD animals receiving i.v. iron loaded Kupffer cells, but no iron was detected in hepatocytes (Figure 8), while in the spleen iron was abundant in both red and white pulp (*Online Supplementary Figure S9*).

ACD/IDA animals receiving oral iron had higher liver and spleen iron concentrations, yet no iron accumulation in hepatocytes or Kupffer cells was detected, while in the spleen minute amounts of the metal were detectable in the white pulp. In contrast, iron accumulated dose-dependently in Kupffer cells and splenic white and red pulp of ACD/IDA animals receiving i.v. iron (Figure 8; *Online Supplementary Figure S9*).

These results show that i.v. but not oral iron supplementation leads to iron accumulation mainly in Kupffer cells in the liver and splenic white pulp macrophages, especially in the presence of inflammation. Of note, in IDA, minute amounts of supplemented iron can also be detected in hepatocytes irrespective of the route of iron administration.

Discussion

To our knowledge this is the first study providing a comparative preclinical analysis of the therapeutic efficacy of oral and i.v. iron preparations for the most common forms of anemia. While oral and i.v. iron supplementation are known to be efficient first-line treatments of IDA,[3,18,25,27] which is also confirmed by our results, the efficacy of iron therapy is less well established in ACD and ACD/IDA. Specifically, the effects of concomitant inflammation on cellular iron transfer, tissue iron distribution and delivery of therapeutically substituted iron for erythroid progenitor cells remained largely elusive.

During inflammation, iron trapping in the reticuloendothelial system is known to be an evolutionary important immune defense strategy to withhold the nutrient iron from invading pathogens.[8,9,39,40] In addition, inflammation-driven hepcidin formation via its interaction with FPN1 impacts on iron absorption from the duodenum and iron recirculation from macrophages.[2,4,8,14,15,41,42] As multiple factors positively, such as inflammation or iron loading, or negatively, such as hypoxia, anemia, erythropoietic hormones and iron deficiency, affect hepcidin expression, the net effect of iron supplementation in anemic individuals with active inflammatory diseases is hard to predict. Moreover, it became evident, not only from different animal models but also from observational studies, that hierarchic networks towards the regulation of hepcidin exist, indicating that anemia, iron deficiency and hypoxia-induced negative regulation may dominate over inflammation-induced hepcidin induction.[2,31,38] However, recent evidence also suggested that, in active tuberculosis, the hormone erythroferrone is unable to suppress inflammation-driven hepcidin expression.[43] Hence, it is not clear yet how inflammatory activity, the degree of anemia and iron availability impact on the therapeutic efficacy of

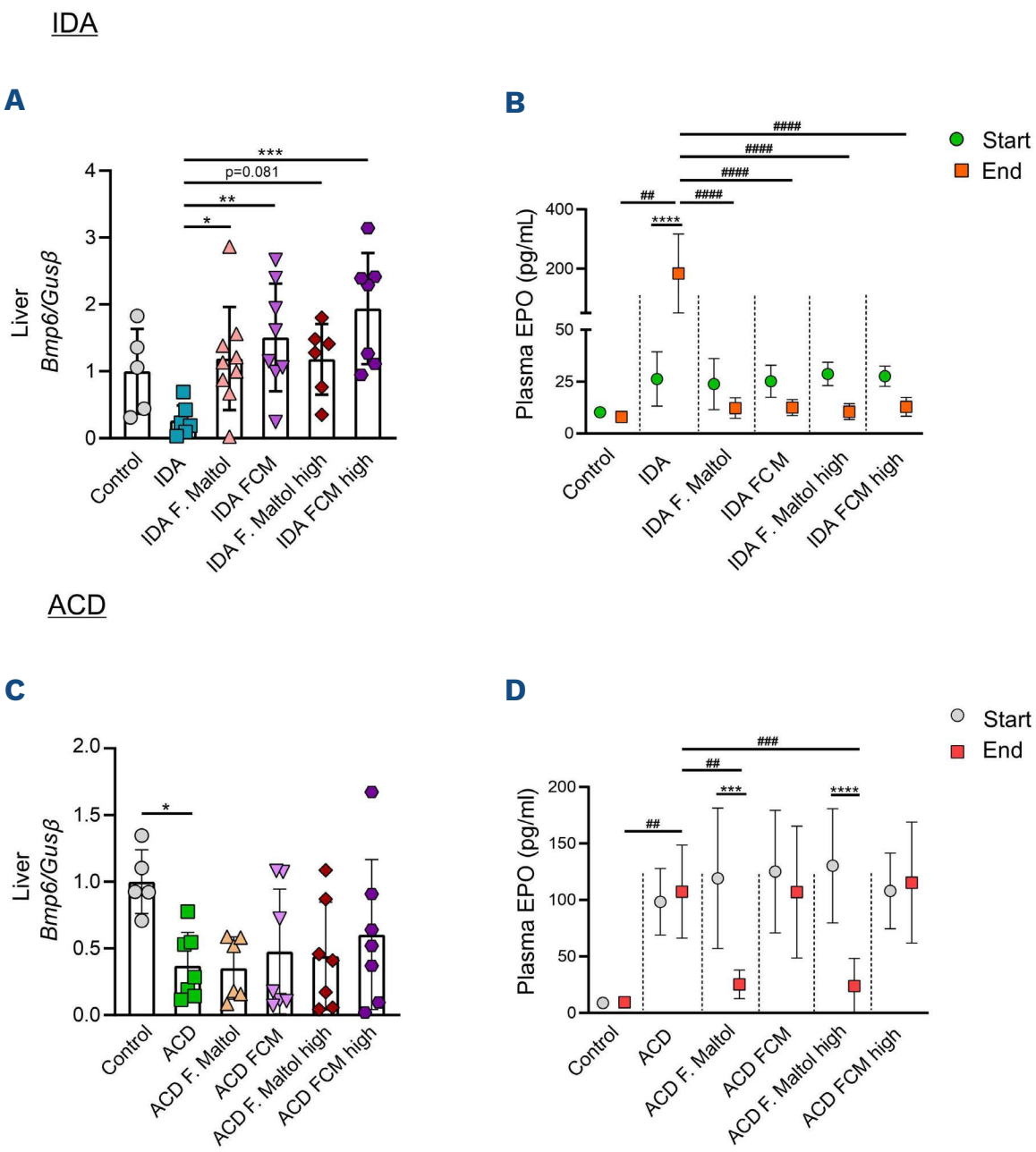

Figure 7. Oral iron supplementation in anemia of chronic disease combined or not with iron deficiency anemia decreases erythropoietin levels, while *Bmp6* mRNA expression increases in iron supplemented animals during iron deficiency anemia. (A) Liver *Bmp6* in iron deficiency anemia (IDA) animals; Gusβ was used as the housekeeping gene. (B) Plasma erythropoietin (EPO) levels in IDA animals at baseline and end of the observation period measured by enzyme-linked immunosorbent assay (ELISA). (C) Liver *Bmp6* in ACD animals; Gusβ was used as the housekeeping gene. (D) Plasma EPO levels in anemia of chronic disease (ACD) animals at baseline and end of the observation period measured by ELISA. (E) Liver *Bmp6* in ACD/IDA animals; Gusβ was used as the housekeeping gene. (F) Plasma EPO levels in ACD/IDA animals at baseline and end of the observation period measured by ELISA. One-way ANOVA with Dunnett´s multiple comparisons test between untreated anemia groups IDA, ACD and ACD/IDA and all other groups was applied respectively for results shown in panels (A, C and E). For results shown in panels (B, D and F) two-way ANOVA with Sidak´s multiple comparisons test was used for analyzing differences between baseline and end of the observation period of each group. **P*<0.05, ***P*<0.01, ****P*<0,001, *****P*<0.0001. The same test was used to analyze differences between untreated anemia groups, respectively IDA, ACD and ACD/IDA, with other groups at end of the experiment. ##*P*<0.01, ###*P*<0.001, ####*P*<0.0001. Results are shown as mean ± standard deviation. Results are from 1 representative experiment with 5-8 animals per group. F Maltol: ferric maltol ; FCM: ferric carboxymaltose.

oral or i.v. iron treatments.

In order to study the principal efficacy of the treatments for replenishing iron stores, we first investigated non-inflamed rats suffering from IDA. We identified that both doses of oral and i.v. iron were efficient in reversing anemia and reducing reticulocytosis. In addition, we found out that i.v. iron was partly stored in the liver and spleen, which fits to ingestion of i.v. iron preparations by macrophages, whereas oral iron is absorbed in the intestine and delivered to different tissues.[8]

These differences in tissue iron distribution were also reflected by partly divergent effects of oral and i.v. iron on regulation of iron metabolism genes in the liver and spleen and tissue iron distribution (Figures 2C and D, 4D

and E, 6D and E and 8; *Online Supplementary Figure S9*). When studying the effects of iron supplementation in ACD with high inflammatory activity, as evidenced by increased levels of IL-6, we found no sustained amelioration of Hb levels. Nonetheless, divergent metabolic effects of oral and i.v. iron were observed. Oral iron supplementation resulted in reduction of inflammatory activity, as reflected by reduced IL-6 levels, increased plasma iron levels, but reduced reticulocyte counts compared to untreated ACD and ACD animals receiving i.v. iron. This indicates at least minimal iron delivery to erythroid progenitor cells, as suggestive from the small increase in Hb levels, the increase of orthochromatic erythroid progenitors and mature RBC numbers in the BM with oral iron therapy over time. Accordingly, we observed reduction of EPO levels with oral iron treatment, suggesting reduced peripheral hypoxia. While hepcidin concentrations in rats receiving oral iron therapy were unchanged, i.v. iron therapy resulted in increasing hepcidin levels over time. This is in line with the observation that hepcidin is a negative predictor of erythrocyte iron incorporation.[44] Previous studies have shown that duodenal iron absorption in the setting of inflammation is reduced due to the action of hepcidin on FPN1 in

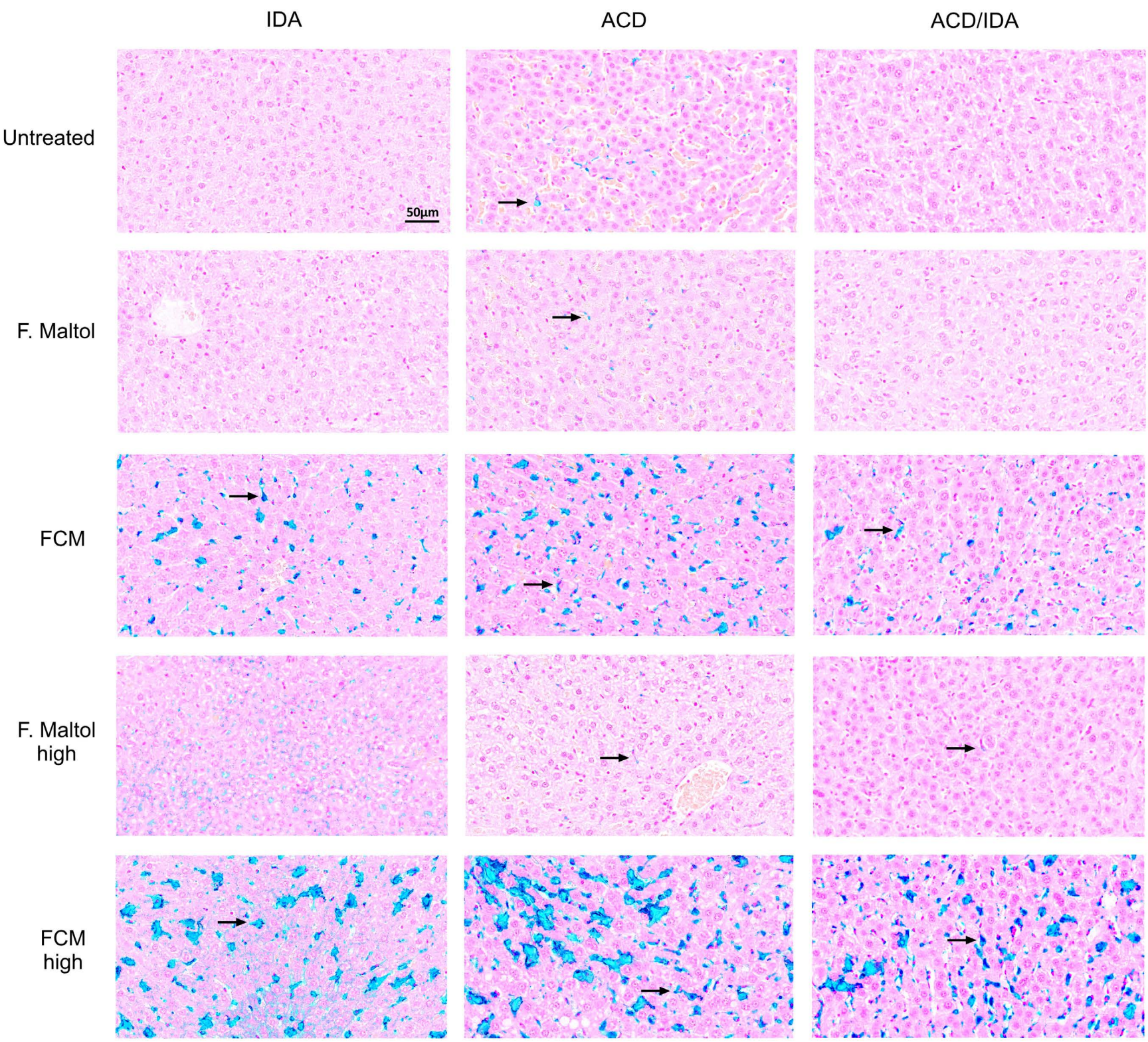

Figure 8. Intravenous but not oral iron treatment leads to iron accumulation in Kupffer cells especially in the presence of inflammation. Liver sections were stained with Prussian blue for iron detection. Blue area corresponds to iron staining (black arrows). Different animal models are shown perpendicularly, untreated and treatment groups horizontally. Scale bar, 50 µm. Results shown are from 1 representative experiment and 1 representative animal per group. An Olympus BX61VS slide-scanner equipped with a 20x objective with a numerical aperture of 0.75, and the OlyVIA software was used for image acquisition.

enterocytes.[14,15] However, our results show that minute amounts of iron are absorbed, subsequently exerting biological effects on erythroid iron delivery, EPO formation and immune regulation.

As a critical hepcidin regulator, *Bmp6* mRNA expression responded to iron treatment diminishing its values in IDA animals. However, its regulation of hepcidin was inconsistent in the presence of inflammation, most likely due to activation of inflammatory signaling pathways affecting the regulatory response of BMP expression to iron availability.[33,45]

This leads to the question of the possible mechanism underlying divergent effects of oral and i.v. iron on inflammatory activity. Iron can affect innate immune effector pathways including the formation of IL-6. While iron supplementation to monocytes *in vitro* negatively impacted on LPS and interferon-γ inducible formation of IL-6 via inhibition of the STAT-1 activation pathway,[46] i.v. iron therapy caused immediate activation of NF-κB driven inflammatory pathways and induction of cytokine expression.[47,48] The differences may be linked to contrasting spatio-temporal cell and tissue distribution of oral *versus* i.v. iron. Oral iron is partly found in hepatocytes, whereas i.v. iron is phagocytosed and likely retained in macrophage endosomes (Figure 8). Of interest, the retention of i.v. iron in Kupffer cells and splenic macrophages was paralleled by an increase in FT expression and tissue iron levels.

When analyzing FPN1 protein levels in liver and spleen, we found that they were increased following i.v. iron therapy, although hepcidin levels were high. As this observation was paralleled by high FT and tissue iron levels, it can be suggested that FPN1 is upregulated transcriptionally and translationally by stress response mechanisms to reduce intracellular iron overload.[8]

When studying the therapeutic efficacy of both iron formulations in ACD/IDA animals, we found that both iron formulations were effective in ameliorating Hb levels and microcytosis (Figure 5A to C). While IL-6 levels were not different between ACD and ACD/IDA animals, hepcidin levels were lower in ACD/IDA, indicating that iron deficiency and/or erythropoietic factors such as EPO or erythroferrone are dominating over the inflammation-induced activation of hepcidin.[15,38,49]

The lower hepcidin formation is in line with the observation of less iron retention in hepatic Kupffer cells in ACD/IDA animals treated with i.v. iron as compared to ACD animals with the same treatment (Figure 8), providing further evidence for the essential role of hepcidin in the control of iron egress from macrophages and iron delivery to erythroid progenitor cells. In parallel, an elegant human study demonstrated that iron release from macrophages is more sensitive to alterations of hepcidin levels than duodenal iron absorption,[28] which supports our observation of iron delivery from oral iron supplements into the body of ACD animals.

The duodenal increase in *Dmt1* mRNA expression seen in IDA and ACD/IDA untreated animals is in agreement with its response to true iron deficiency.[35,50] Of interest, in subjects with ACD and ACD/IDA with iron treatment, we observed no association of DMT1 levels with hepcidin concentrations, suggesting that in addition to hepcidin other mechanism such as inflammatory cytokines, enterocyte iron content and/or hypoxia inducible factors affect DMT1 expression.[51-54]

We also observed differences in erythroid progenitor cell differentiation between the different types of anemia. Strikingly, pro-erythroblast numbers were significantly induced in IDA and ACD/IDA animals, but not in ACD rats suggesting that pro-erythroblast expansion is driven by iron deficiency rather than by reduced Hb levels, and that it is independent of inflammation, as it was comparable between IDA and ACD/IDA animals.

In summary, we provide evidence of differences in iron distribution and metabolic effects between oral and i.v. iron therapy. We also show that both iron therapies can ameliorate IDA and ACD/IDA, whereas they remain largely ineffective in correcting ACD in the setting of ongoing inflammatory activity, which is in line with the observation of therapeutic inefficacy of iron supplementation in intensive care unit patients.[55] Of note, i.v. iron is partly retained in hepatic and splenic macrophages, which is paralleled by increased expression of hepcidin and IL-6, whereas oral iron supplementation was linked to reduced IL-6 formation and slightly ameliorated erythropoiesis.

Whether our findings are specific for the iron sources used, i.e., F. Maltol and FCM, or also applies for other formulations, remains to be shown. We hypothesize that iron-carbohydrate complexes like i.v. iron dextran and iron dextrin would have the same effect as FCM, and that the outcome seen with F. Maltol may be partially linked to its non-salt-based ferric iron formulation, but may also largely apply to other oral iron formulations. Of interest, in our models oral iron was provided once daily and the question remains whether iron administration on alternate days could improve outcome.[56]

It raises the question whether physicians should be especially cautious when treating ACD associated with ongoing inflammation and contemplate if patients will benefit from iron therapy in that setting.[55]

These contrasting therapeutic effects of iron treatment urge the identification of easy-to-use biomarkers that can clearly differentiate between ACD and ACD/IDA, in order to provide the best therapeutic effects in patients. It will also be of interest to see if combination treatment of ACD with erythropoiesis-stimulating agents can improve iron delivery to erythroid progenitor cells by increasing TFR1 expression, EPO signaling via Scribble or by reducing inflammatory activity.[57-59]

Disclosures

GW received honoraria for lectures and advisory board participation from Vifor and Pharmacosmos, two manufacturers of iron compounds. GW is the director of the Christian Doppler Laboratory for Anemia and Iron Metabolism Research. IT consults for Kymab, Cambridge.

Contributions

LVdS and GW planned and designed the project, analyzed and interpreted data and wrote the manuscript. LVdS, AH, CF, VP and MA did critical animal handling during the course of the experiments. LVdS, AH, CF, VP, MA, IT, PT, MS, NB, RH, ED, LvR, SB, MB-P performed experiments. GW was responsible for the supervision, conceptualization, and funding acquisition. All authors read and approved the final manuscript.

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Data-sharing statement

The data are available from the first author upon reasonable request.

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