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

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Supplemental material and methods

Animals

Rats were kept on an adequate iron diet (50 mg Fe/kg, Ssniff, Germany) during the whole course of the experiment, unless otherwise specified elsewhere. The animals had free access to food and water and were kept according to institutional and governmental guidelines in the animal housing unit of the Medical University of Innsbruck with a 12-hour light-dark cycle and an average temperature of 20°C plus or minus 1°C. 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).

Ferric Carboxymaltose (Ferrinject ®) (FCM) was chosen as i.v. iron source as it is an established treatment for correcting iron deficiency in patients with different underlying diseases.[1, 2] In previous studies with rodents, dosages of up to 90 mg/kg per week and even 1000 mg/kg as a single dose were used.[3] For our studies, we used an initial dose of 15mg/kg which closely applies to the recommendations for dosing in humans.[1] Groups receiving this dose were called FCM. To analyze the aspects of a higher dose administration and rule out a limitation of therapeutic efficacy due to insufficient iron supplementation, we also studied a higher dose of 45mg/kg. Groups receiving this dose were called FCM high. FCM was administered intravenously in the tail vein, once a week for the entire duration of the experiment.

Ferric Maltol (Feraccru ®), a carbohydrate iron formulation, was used as oral iron therapy as it has been demonstrated efficient to treat iron deficiency also associated with inflammatory diseases such as intestine bowel disease (IBD)[4, 5] or in anemic patients with pulmonary hypertension. [5, 6] Its daily dose and frequency of application in humans vary [4] and in previous experiments with rodent models dosages up to 50mg/kg have been used.[7] As only a part of supplemented oral iron is absorbed[8] we used either 5mg/kg or 15mg/kg as doses for our study, administered by oral gavage, once a day. Groups were called F. Maltol and F. Maltol high, respectively.

The iron deficiency anemia (IDA) model

For generating animals with IDA (Figure S1A), 6 weeks old Lewis rats were kept on a low iron diet (<9mg Fe/kg) during the whole course of the experiment. In addition, animals were phlebotomized by withdrawing 1.8ml of blood daily for 5 consecutive days (week 1).[9] Rats were randomized into treatment groups or control. To this point, we aimed to maintain IDA for the whole time of treatment. On weeks 2 and 3, 1.8ml of blood were drawn twice a week on days 3, 5, 9 and 11, and the same amount once a week in weeks 4 and 5, on days 19 and 25. Oral iron supplementation (either 5mg/kg or 15mg/kg) started on day 1 and was administrated daily by oral gavage (OG) for four weeks. I.v. iron (either 15mg/kg or 45mg/kg) was supplemented once a week, on days 4, 10, 16 and 23. The experiment was terminated with euthanasia of all animals on day 29.

The anemia of chronic disease (ACD) model

For these experiments, we used an established ACD rat model (Figure S1B) [9]. Lewis rats, 6 weeks old, were injected with purified group A streptococcal peptidoglycan-polysaccharide (PG-APS) resulting in the development of inflammatory arthritis and anemia with characteristics of ACD (Hg<13.5 mg/dl and granulocyte count > 60%).[10] After three weeks,

rats were randomized into treatment groups or control. Iron supplementation lasted for 4 weeks, from day 1 to day 28. Oral iron was administrated once daily by OG in two different doses (either 5 or 15mg/kg). I.v. iron was administrated once a week, on days 3, 10, 17 and 24, also in two different doses (either 15mg/kg or 45mg/kg). On day 29, the experiment was terminated.

The anemia of chronic disease combined with iron deficiency anemia (ACD/IDA) model

For generating rats with combined ACD and IDA, we used a model described previously[9, 11, 12] (Figure S1C). Lewis rats, 6 weeks old, were injected with PG-APS and developed features of arthritis with ACD (Hg<13.5 mg/dl and granulocytes count > 60%) after two weeks. At this point, a group of animals was randomized into ACD control. The remaining animals were then fed a low iron diet (<9mg Fe/kg) and phlebotomized daily from day 1 to day 5 (week 3) with dewing 1.8ml of blood each time. After that week, rats had typical features of ACD/IDA.[9] Animals were then randomized into control or treatment groups. Since we aimed to maintain the combination of ACD/IDA during the whole time of treatment, phlebotomy was further performed once a week on days 5, 12 and 19. Iron supplementation then lasted for a total of 3 weeks. Oral iron supplementation (either 5mg/kg or 15mg/Kg) started on day 1 after randomization and was applied by OG once daily until day 21. I.v. iron (either 15mg/kg or 45mg/kg) was supplemented once a week, on days 3, 10 and 17. The experiment was terminated with euthanasia of all animals on day 22.

Analysis of blood parameters

Blood counts were determined using a Vet-ABC Animal blood counter (Scil animal care company GmbH, Viernheim, Germany). Plasma iron was measured using the QuantiChrom iron assay kit (BioAssay Systems, Hayward, CA) according to the manufacturer's instructions. Plasma hepcidin was determined using the hepcidin-25 (rat) enzyme immunoassay kit (Peninsula Laboratories International, Inc., San Carlos, CA, USA) according to the manufacturer's protocol. IL-6 plasma levels were measured using the Rat IL-6 Quantikine ELISA Kit (cat. no. R6000B, R&D Systems, Inc.), following the supplier's guidelines (R&D Systems, Minneapolis, MN, USA). Erythropoietin (EPO) plasma levels were determined using the LEGEND MAX™ Rat Erythropoietin ELISA Kit (cat. no. 442807, Biolegend, Inc., San Diego, CA, USA), according to the suppliers´ protocol.

RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was prepared from liquid nitrogen-frozen rat and mouse tissues using acid guanidinium thiocyanate-phenol-chloroform extraction with peqGOLD Tri-Fast™ (Peqlab, Germany). For reverse transcription 4µg RNA was used. TaqMan real-time PCR was performed at least in duplicates on a CFX96 light cycler (Bio-Rad). Ssofast Probes Supermix and Ssofast EvaGreen Supermix (Bio-Rad Laboratories GmbH, Vienna, Austria) were used according to the manufacturer's instructions. Real-time PCR reactions were performed on a CFX Cycler and analyzed with CFX software (BioRad). Gene expression was normalized using ΔΔct calculations.

The following TaqMan PCR primers and probes were used:

Rat *Hamp*: 5'-TGAGCAGCGGTGCCTATCT-3', 5'-CCATGCCAAGGCTGCAG-3', FAM-CGGCAACAGACGAGACAGACTACGGC-BHQ1

Rat *Gusβ* (beta-glucoronidase): 5'-ATTACTCGAACAATCGGTTGCA-3', 5'-GACCGGCATGTCCAAGGTT-3', FAM-CGTAGCGGCTGCCGGTACCACT-BHQ1

Rat *Fpn1*: 5'-TTGGTGACTGGGTGGATAAGAA-3', 5'-CCGCAGAGAATGACTTTC-3', FAM-CAGACTTAAAGTGGCCCAGACGTCCCTG-BHQ1

Rat *Ncoa4*: 5'-TAGAGAGACACTTCTGAGGTGCAGTGATG-3', 5'-TTTCATTTGCTGCTCGGCCC-3'

Rat *Tfr1*: 5'-ATGAGGAACCAGACCGCTACA-3', 5'-CCACACTGGACTTCGCAACA-3', FAM-CCAAGCGTCTCTCTGGGCTCCTACTACA-BHQ1

Rat *Dmt1*-IRE: 5´GTT CTA CTT GGG TTG GCA GTG TTT3´, 5´CTC AGC AGG ACT TTA GAG ATG CTT AC3´, FAM-ACA GTC CAG GAA CGA CAG GCC CAA-BHQ1

Rat *Bmp6*: 5'-TGA GCT TTG TGA ACC TGG TG-3', 5'-CTC GGG AAT CTG GGA TAA GTT G-3'

Western blot and tissue iron determination

Protein extraction and western blotting were performed as described previously (12). Protein extracts were prepared from nitrogen frozen tissue homogenized in cytoplasmic lysis buffer (25 mM Tris-HCl pH 7.4, 40 mM KCl, 1% Triton X-100) containing 1 µg/ml of each aprotinin, leupeptin and phenylmethylsulfonyl fluoride.

For Western blotting, 20 mg of protein extracts were run either on a 10% (for TfR1 and Fpn1) or a 15% (for ferritin and β-actin) SDS-polyacrylamide gel. Proteins were transferred onto a nylon membrane (Hybond-P, Amersham-Pharmacia, Vienna, Austria) and blocked in 1xTBS buffer containing 5% dry milk and 0.1% Tween (Merck, Vienna, Austria). The membrane was incubated either with human anti-TfR1-antibody (0.5 µg/ml, Zymed, Vienna, Austria), human anti-ferritin-antibody (2 µg/ml, Dako, Vienna, Austria), rabbit anti-rat-ferroportin-antibody, rabbit anti-NCOA4 antibody (1mg/ml, Abcam, Austria) or human anti-actin (2 µg/ml, Sigma, Germany) and further processed as described.[13] Actin hybridization was used to demonstrate equal protein loading onto gels.

For quantification, densitometry data were acquired on a ChemiDoc Touch Imaging System (Bio-Rad) and analyzed with Quantity One software (Bio-Rad).

Tissue iron content was determined with acid-hydrolysed tissue homogenates with a colorimetric method employing bathophenanthroline disulfonic acid and L-ascorbic acid in a sodium acetate assay buffer. The calculated iron quantity was normalized to the wet tissue weight for each sample.[13]

Flow cytometry analysis

PE-anti-CD71 (clone OX-26, BD), eFluor 450-anti-CD45 (clone OX1, eBioscience), FITC-anti-CD44 (Thermo Fisher), APC-anti-rat erythroid cells (REC) (clone HIS49, BD) were used for analysis of erythroid progenitor subpopulations.

Gating strategy and analysis procedure of rats BM cells were performed as described [12, 13] (Figure S2A): BM cells were stained with antibodies against CD45, CD44, CD71 and anti-erythroid cells. DAPI was used to exclude dead cells. After exclusion of doublets and multiplets using the FSC-A and FSC-H, the CD45-low DAPI-negative population was identified. This population was then gated with CD44 on the y-axis and REC on the x-axis. Proerythroblasts

were characterized as REC-middle CD44-high. REC-high population was plotted according to FSC on the x-axis and CD44 expression on the y-axis, which resulted in the separation of the 4 different stages of erythroid differentiation, corresponding to basophilic erythroblasts (II), polychromatic erythroblasts (III), orthochromatic erythroblasts (IV) and mature RBC (V).

The quantification of peripheral blood reticulocytes was carried out as described previously [12] (Figure S2B): Thiazol orange (TO) was used as a dye. After ecluding doublets and multiplets using the FSC-A and FSC-H, peripheral reticulocytes were identified as TO positive cell population.

Immunohistochemistry

Livers were dissected and snap-frozen in Tissue Tek-OCT embedding compound (VWR) and stored at -80°C. Tissue sections of a thickness of 5µm were desiccated, fixed with 4% paraformaldehyde for 20 min at room temperature, blocked with 5% normal horse serum at room temperature for 30 min and incubated with Alexa Fluor 594 Phalloidin (Thermo Fisher Scientific) as well as co-stained with Alexa Fluor 647 anti-mouse Clec4f-antibody for 1 h at 4°C (1:1000 Biolegend). Finally, cover slips were mounted with fluorescence mounting medium (DAKO) containing DAPI (Thermo Fisher Scientific) for DNA staining. For image acquisition, an Olympus BX61VS slide-scanner equipped with a 20x objective with a numerical aperture of 0.75, and the OlyVIA software (Olympus) were used.

Prussian blue staining

Sections (5µm) were incubated with ammonium sulfide solution 20% (Sigma) for 1min and then washed for 10s with running tap water. Then, sections were incubated for 1min with 10% potassium hexacyanoferrate (Carl Roth) mixed with 0.5 M hydrochloric acid (Sigma Aldrich) and washed again with running tap water for 10s. Microscope slides were dyed using nuclear fast red solution (Gatt-Koller) for 5min. After an additional washing step (running tap water for 10s), sections were incubated in 100% acetone for 10min at 4°C, followed by 96% Ethanol for 2min and afterwards ethanol absolute. Then, sections were drained in xylene for 2min and mounted with mounting medium Entellan (Sigma Aldrich). For image acquisition, an Olympus BX61VS slide-scanner equipped with a 20x objective with a numerical aperture of 0.75, and the OlyVIA software were used. Prussian blue stained tissue area was analyzed according a to previously published protocol.[14]

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Figure S1

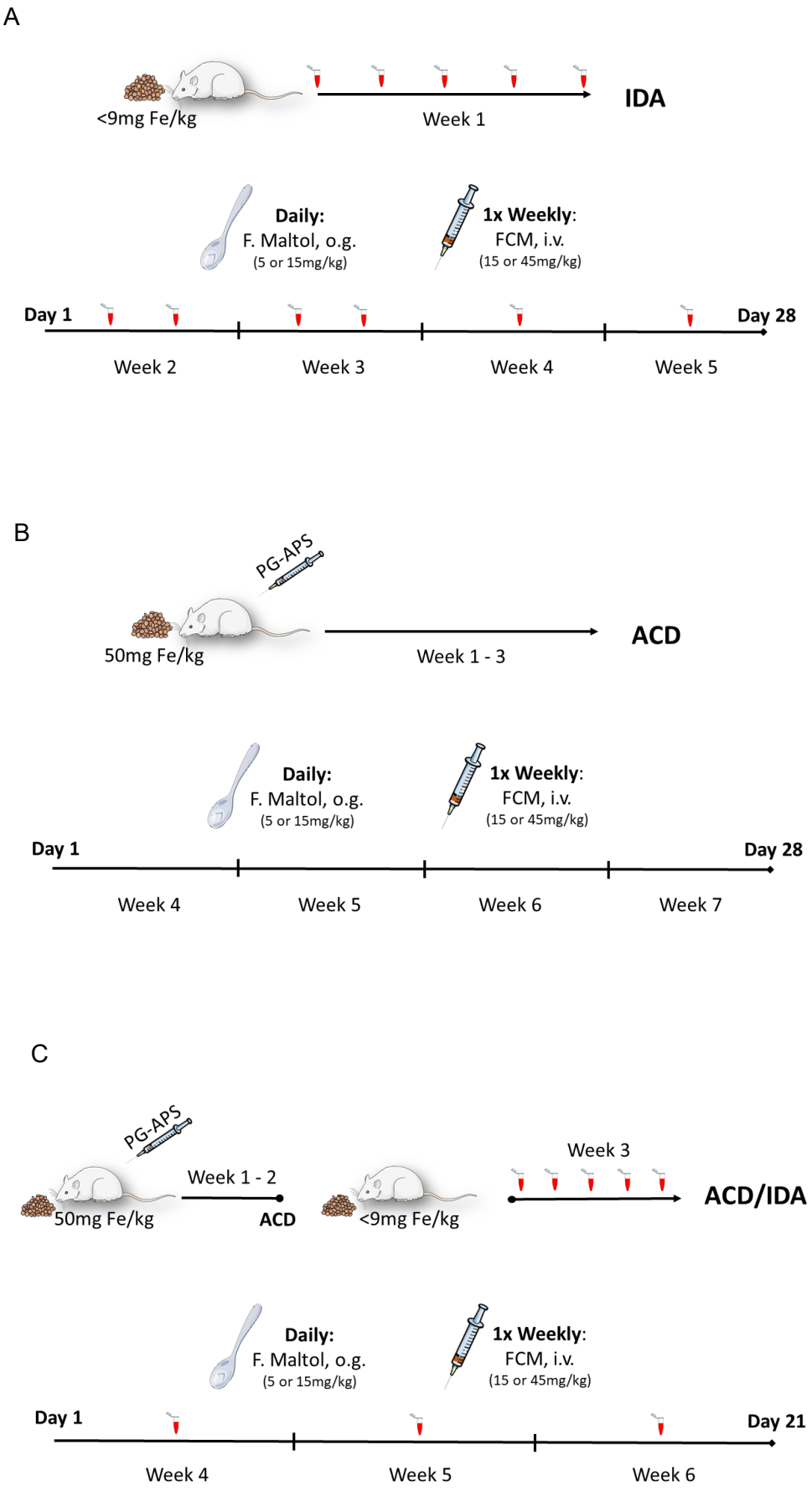

A
<9mg Fe/kg
Week 1
IDA
Daily:
F. Maltol, o.g.
(5 or 15mg/kg)
1x Weekly:
FCM, i.v.
(15 or 45mg/kg)
Day 1
Day 28
Week 2
Week 3
Week 4
Week 5
B
PG-APS
50mg Fe/kg
Week 1 - 3
ACD
Daily:
F. Maltol, o.g.
(5 or 15mg/kg)
1x Weekly:
FCM, i.v.
(15 or 45mg/kg)
Day 1
Day 28
Week 4
Week 5
Week 6
Week 7
C
PG-APS
Week 1 - 2
50mg Fe/kg
ACD
Week 3
<9mg Fe/kg
ACD/IDA
Daily:
F. Maltol, o.g.
(5 or 15mg/kg)
1x Weekly:
FCM, i.v.
(15 or 45mg/kg)
Day 1
Day 21
Week 4
Week 5
Week 6

Figure S2

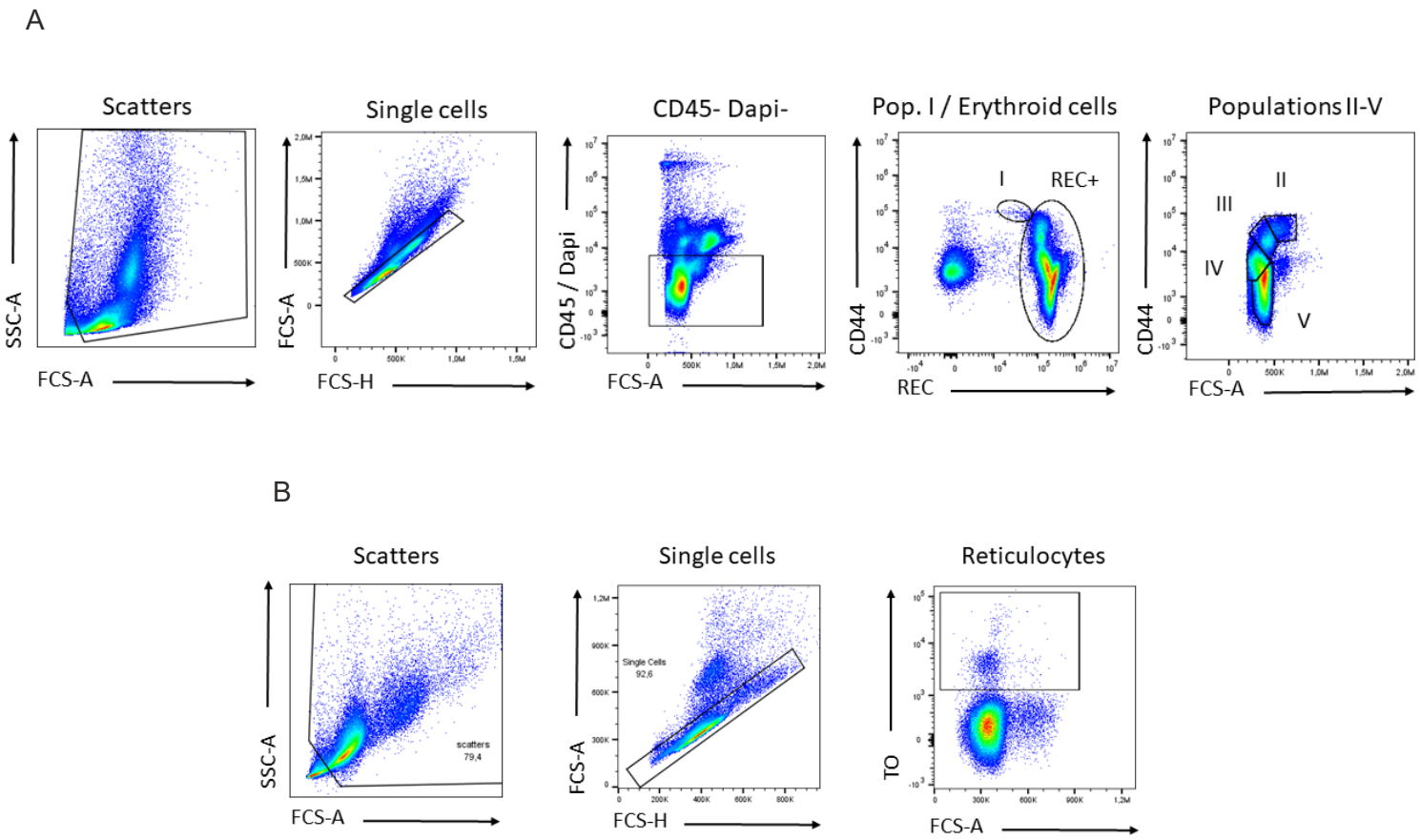


Figure S3

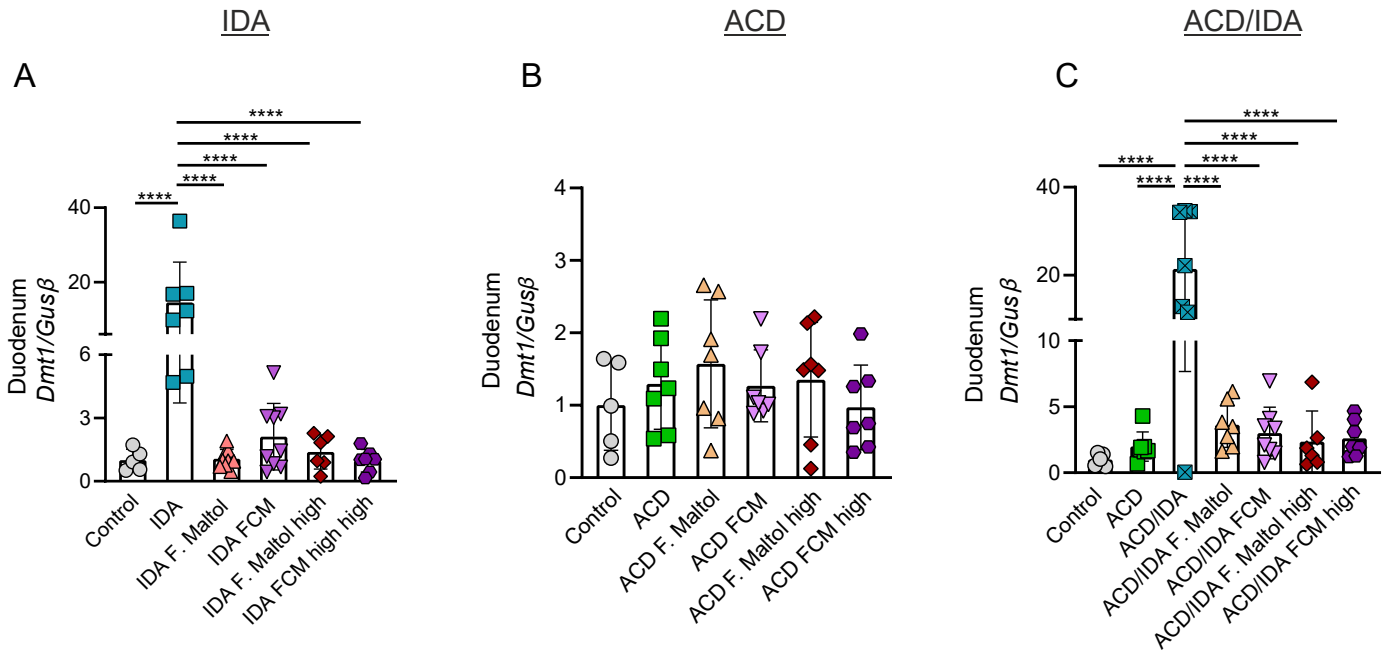
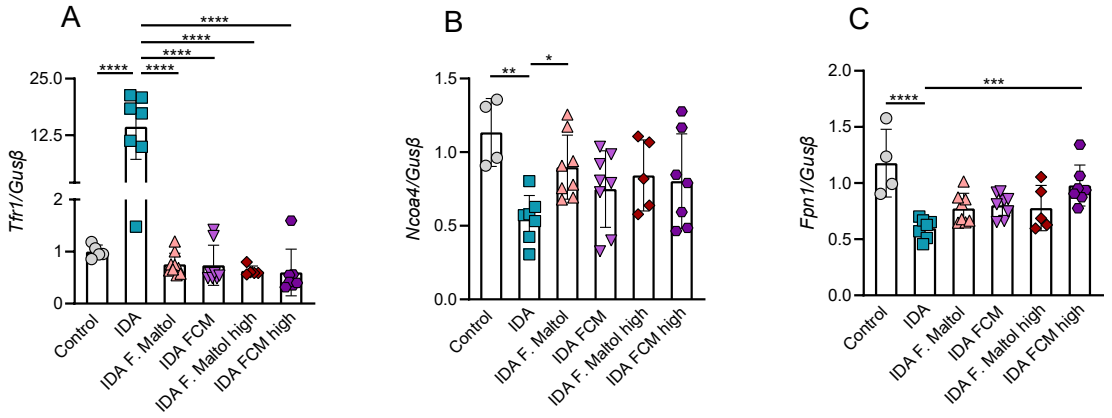
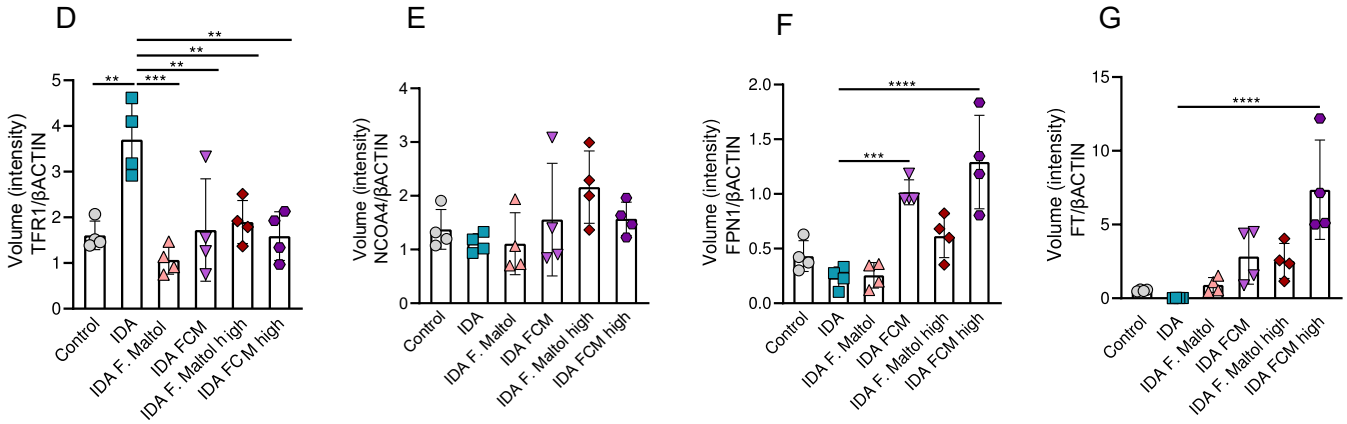


Figure S4

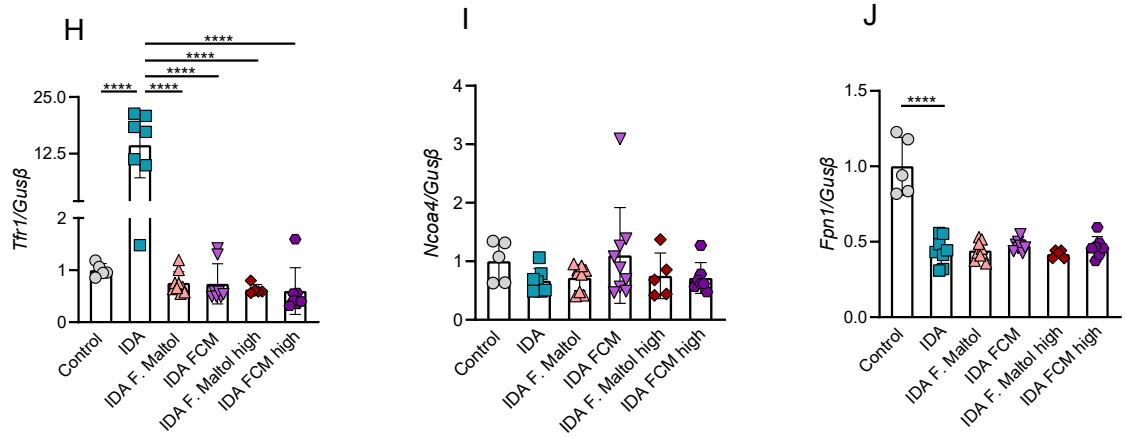
IDA Liver RTPCR



IDA Liver WB quantification



IDA Spleen RTPCR



IDA Spleen WB quantification

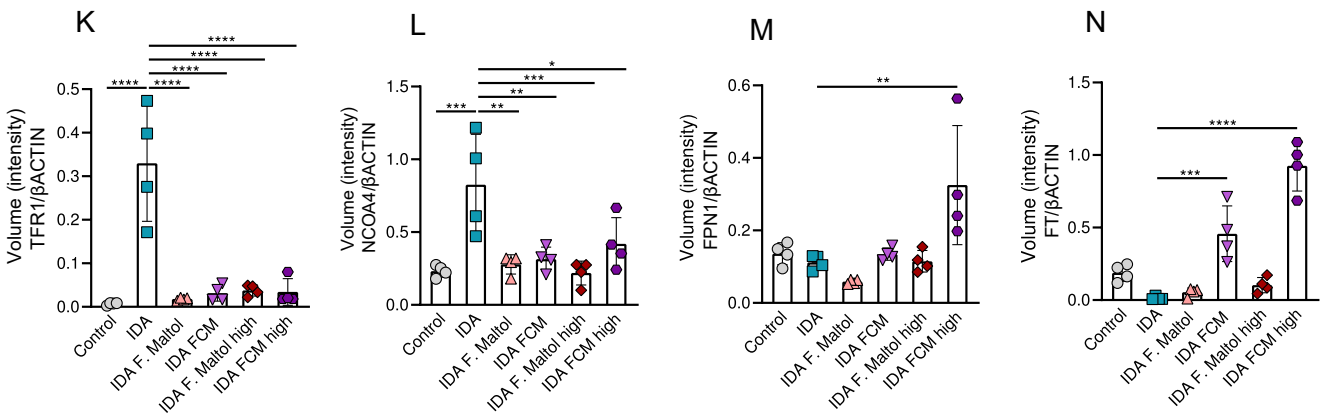
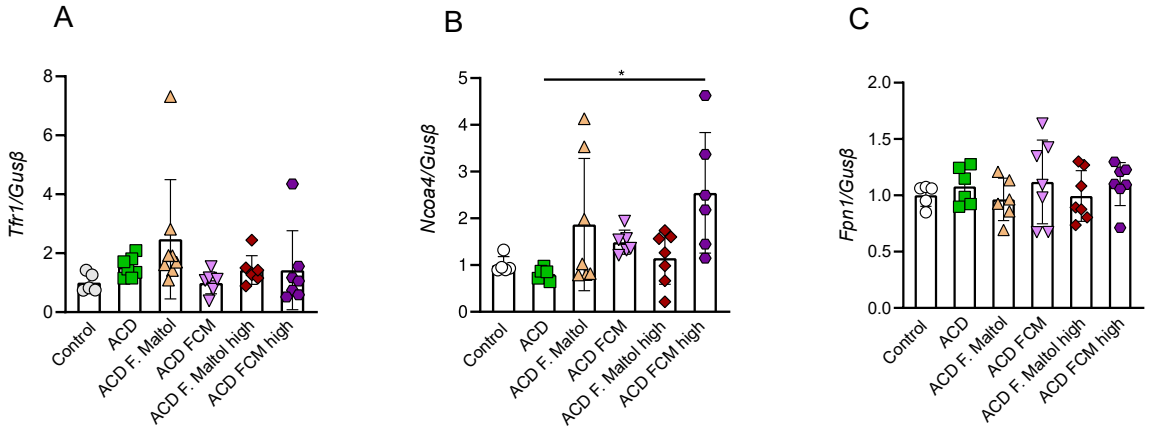
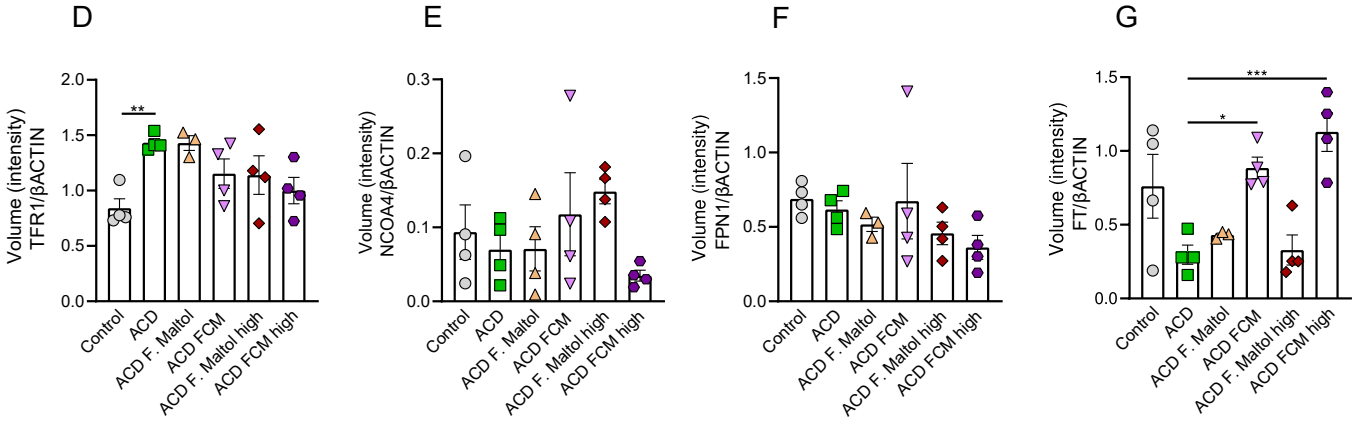


Figure S5

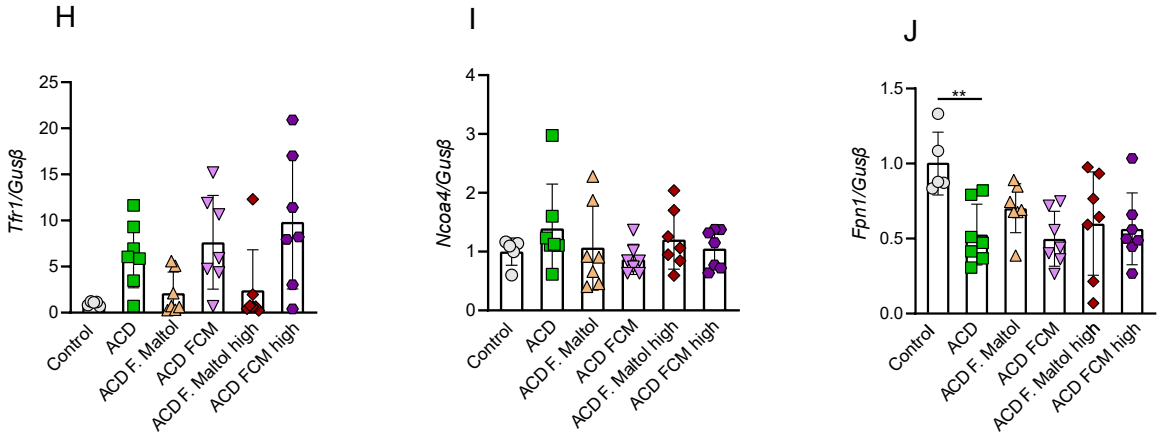
ACD Liver RTPCR



ACD Liver WB quantification



ACD Spleen RTPCR



ACD Spleen WB quantification

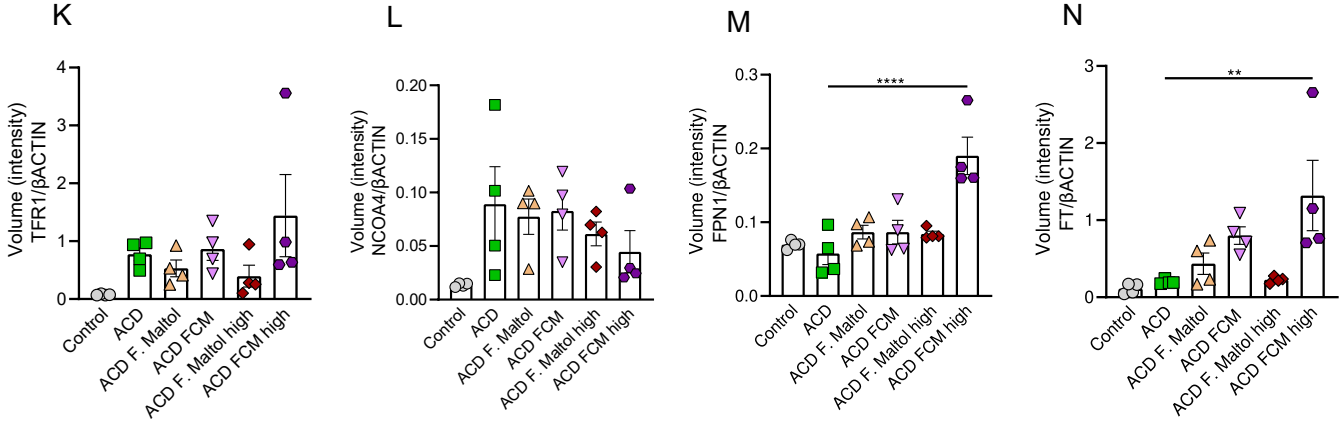
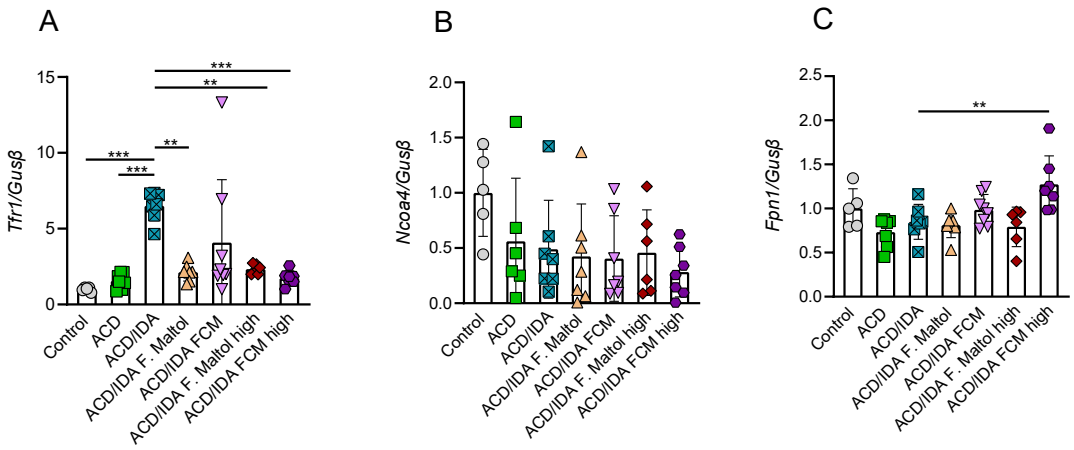
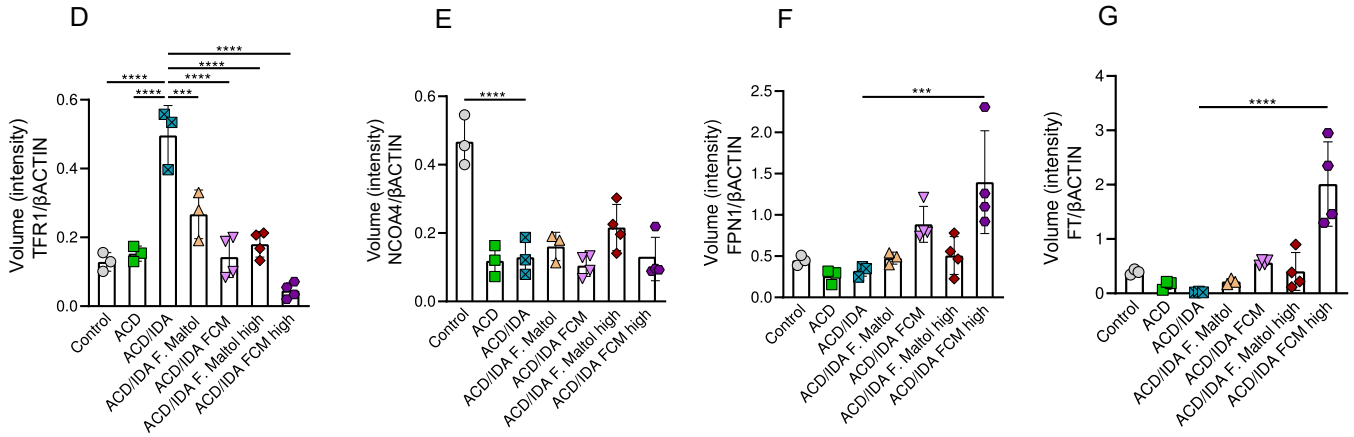


Figure S6

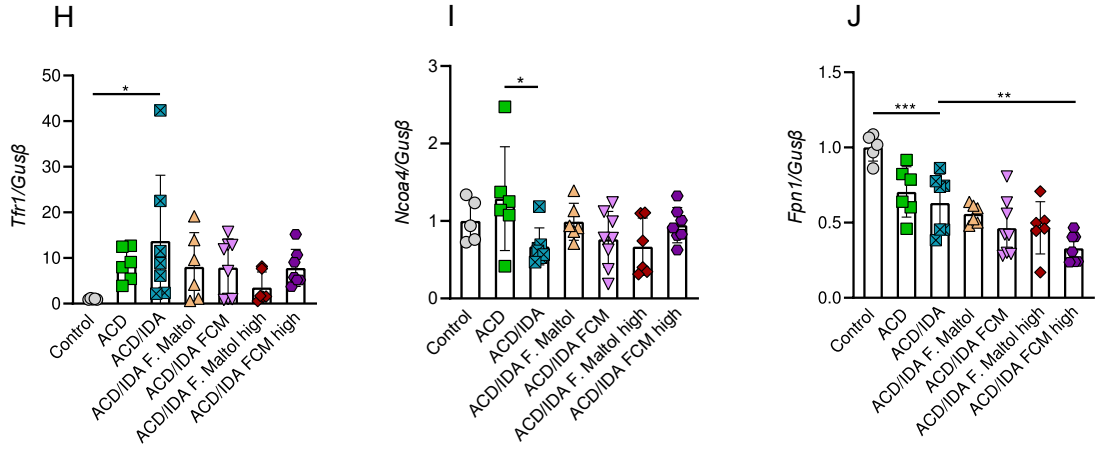
ACD/IDA Liver RTPCR



ACD/IDA Liver WB quantification



ACD/IDA Spleen RTPCR



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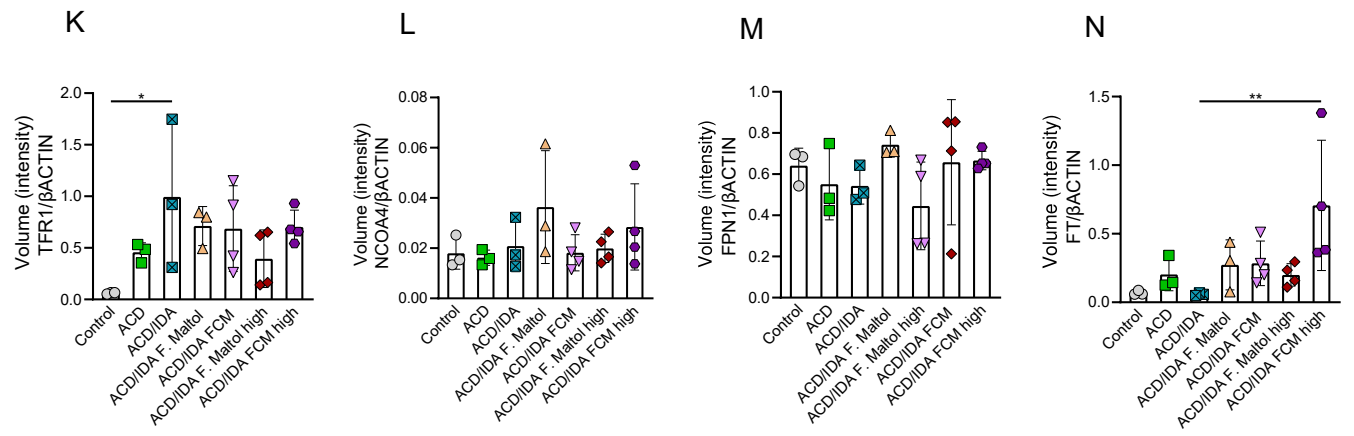


Figure S7

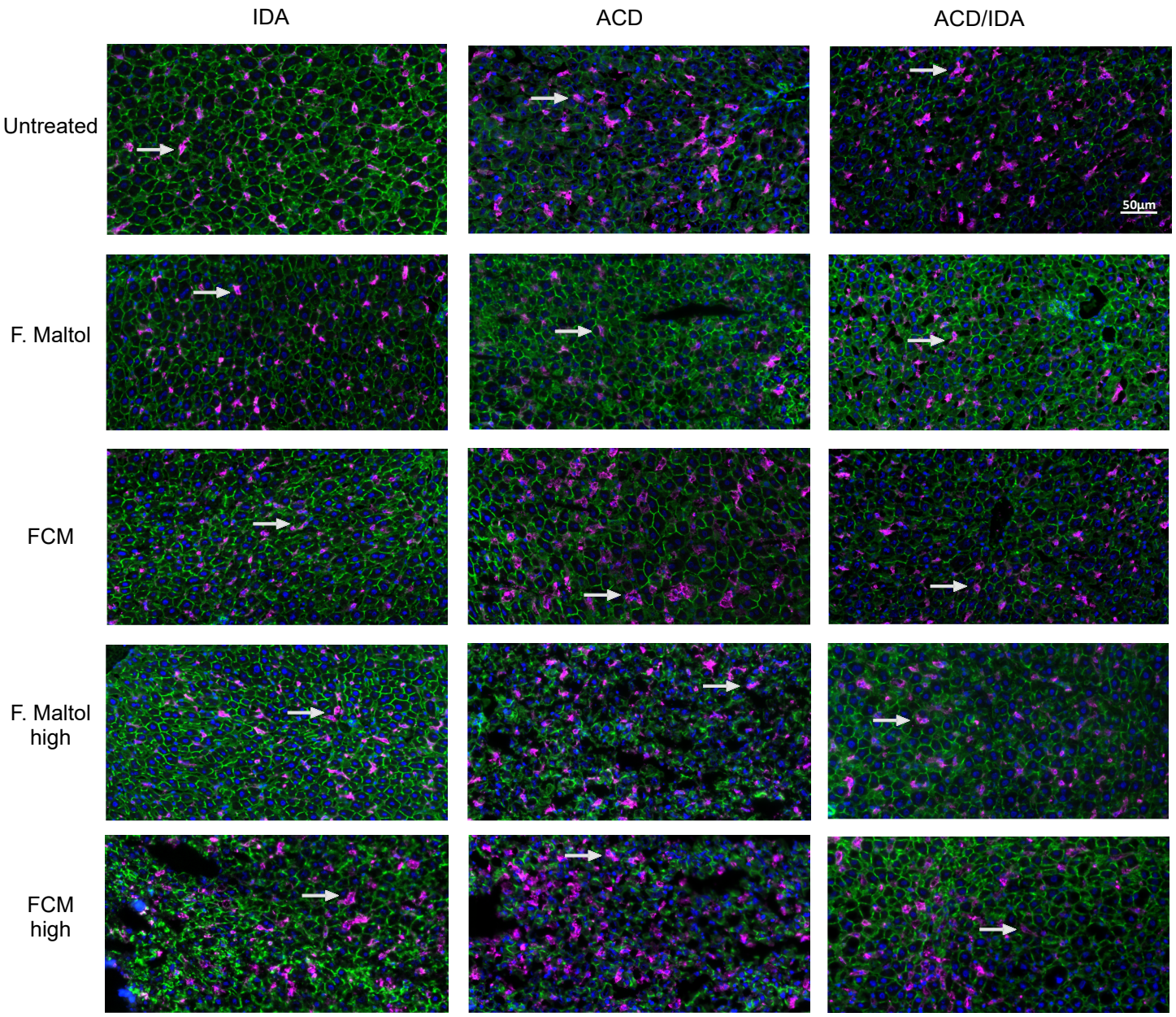


Figure S8

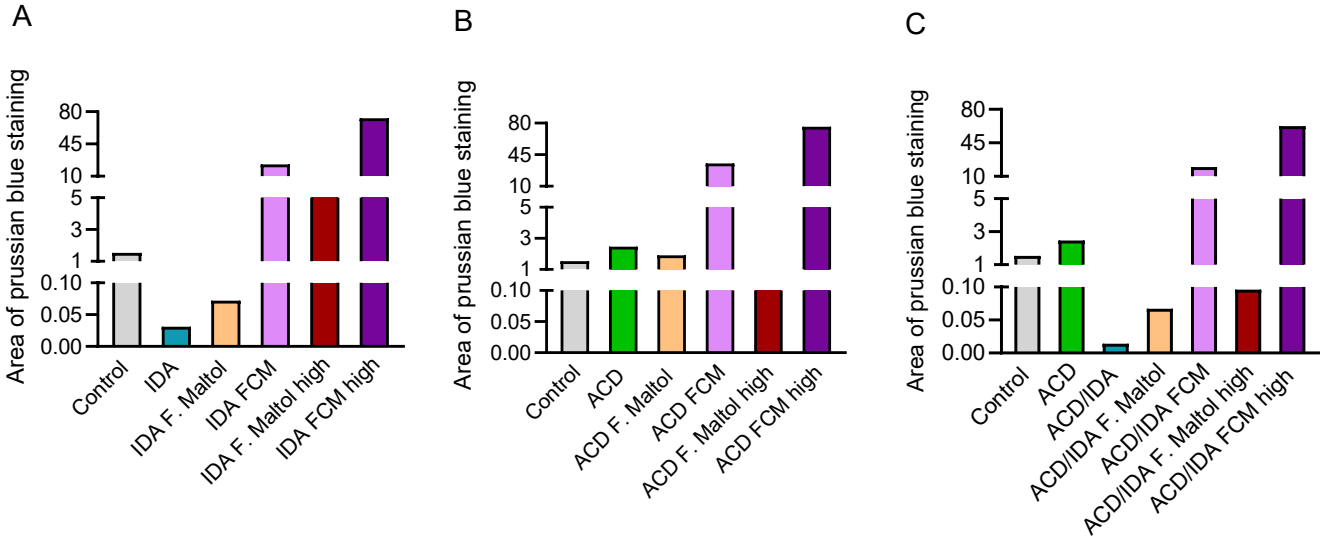
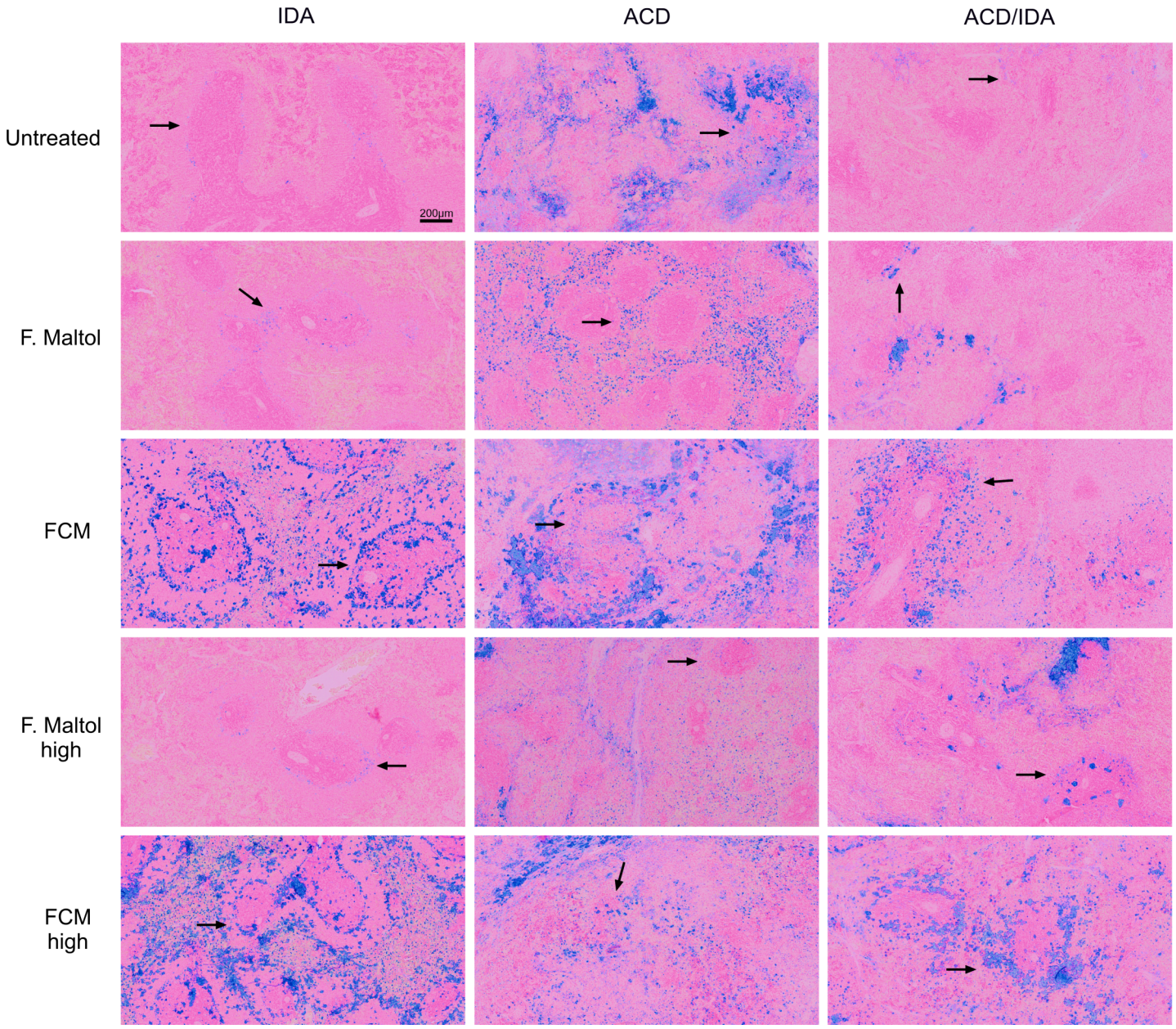


Figure S9


IDA
ACD
ACD/IDA
Untreated
F. Maltol
FCM
F. Maltol high
FCM high
200µm

Legends to the supplemental figures

Figure S1. Experimental setup of the animal models. (A-C) Lewis rats were used to create previously stablished methods of iron deficiency anemia (IDA), anemia of chronic disease (ACD) and a combination thereof ACD/IDA (details in methods). Experimental setup of (A) IDA model, (B) ACD model and (C) ACD/IDA model. Figures S1A - C were created using Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Figure S2. Gating strategy of flow cytometry analyses. (A-B) Flow cytometry was used to analyze erythroid progenitors' populations in the bone marrow (BM) and peripheral blood reticulocytes. (A) Illustrated gating strategy of erythroid progenitor populations in the BM. Population I consists of proerythroblasts, population II of basoerythroblasts, population III of polyerythroblasts, population IV of orthroerythroblasts and population V of mature red blood cells. (B) Illustrated gating strategy of peripheral blood reticulocytes. More details are described in the methods.

Figure S3. Real time polymerase chain reaction (RTPCR) of duodenum in the different anemia models. (A-C) Duodenal RTPCR. *Dmt1* mRNA expression was analyzed in the duodenum of (A) iron deficiency anemia (IDA), anemia of chronic disease (ACD) and anemia of chronic disease combined with iron deficiency anemia (ACD/IDA) models. *Gusβ* was used as housekeeping gene. One-way ANOVA with Dunnett´s multiple comparisons test between the respective untreated anemia groups and all other groups was applied. Results are shown as mean ± SD. *p<0.05, **p<0.01, ***p<0,001, ****p<0.0001. Results are from one representative experiment consisting of 5-8 animals per group.

Figure S4. **Western blot (WB) quantification and real time polymerase chain reaction (RTPCR) of liver and spleen in the iron deficiency anemia (IDA) model.** (A-C) Liver RTPCR. Liver (A) *Tfr1*, (B) *Ncoa4*, (C) *Fpn1* mRNA levels. *Gusβ* was used as housekeeping gene. (D-G) Liver WB quantification. Volume intensity of (D) TFR1/βACTIN, (E) NCOA4/βACTIN, (F) FPN1/βACTIN, (G) FT/βACTIN. βACTIN was used as housekeeping protein. (H-J) Spleen RTPCR. Spleen (H) *Tfr1*, (I) *Ncoa4*, (J) *Fpn1* mRNA levels. *Gusβ* was used as housekeeping gene. (K-N) Spleen WB quantification. Volume intensity of (K) TFR1/β-ACTIN, (L) NCOA4/βACTIN, (M) FPN1/βACTIN, (N) FT/βACTIN. βACTIN was used as housekeeping protein. One-way ANOVA with Dunnett´s multiple comparisons test between IDA and all other groups was applied. Results are shown as mean ± SD. *p<0.05, **p<0.01, ***p<0,001, ****p<0.0001. Results are from one representative experiment consisting of 5-8 animals per group.

Figure S5. Western blot (WB) quantification and real time polymerase chain reaction (RTPCR) of liver and spleen in the anemia of chronic disease (ACD) model. Liver RTPCR levels. Liver (A) *Tfr1*, (B) *Ncoa4*, (C) *Fpn1* mRNA levels. *Gusβ* was used as housekeeping gene. (D-G) Liver WB quantification. Volume intensity of (D) TFR1/βACTIN, (E) NCOA4/βACTIN, (F) FPN1/βACTIN, (G) FT/β-ACTIN. βACTIN was used as housekeeping protein. (H-J) Spleen RTPCR. Spleen (H) *Tfr1*, (I) *Ncoa4*, (J) *Fpn1* mRNA levels. *Gusβ* was used as housekeeping gene. (K-N) Spleen WB quantification. Volume intensity of (K) TFR1/βACTIN, (L) NCOA4/βACTIN, (M) FPN1/βACTIN, (N) FT/βACTIN. βACTIN was used as housekeeping protein. One-way ANOVA with Dunnett´s multiple comparisons test between ACD and all other groups was applied. Results are shown as mean ± SD. *p<0.05, **p<0.01, ***p<0,001, ****p<0.0001. Results are from one representative experiment consisting of 5-8 animals per group.

Figure S6. Western blot (WB) quantification and real time polymerase chain reaction (RTPCR) of liver and spleen in the anemia of chronic disease combined with iron deficiency anemia (ACD/IDA) model. Liver RTPCR. Liver (A) *Tfr1*, (B) *Ncoa4*, (C) *Fpn1* mRNA levels. *Gusβ* was used as housekeeping gene. (D-G) Liver WB quantification. Volume intensity of (D) TFR1/βACTIN, (E) NCOA4/βACTIN, (F) FPN1/βACTIN, (G) FT/βACTIN. βACTIN was used as housekeeping protein. (H-J) Spleen RTPCR. Spleen (H) *Tfr1*, (I) *Ncoa4*, (J) *Fpn1* mRNA levels. *Gusβ* was used as housekeeping gene. (K-N) Spleen WB quantification. Volume intensity of (K) TFR1/βACTIN, (L) NCOA4/βACTIN, (M) FPN1/βACTIN, (N) FT/βACTIN. βACTIN was used as housekeeping protein. One-way ANOVA with Dunnett´s multiple comparisons test between ACD/IDA and all other groups was applied. Results are shown as mean ± SD. *p<0.05, **p<0.01, ***p<0,001, ****p<0.0001. Results are from one representative experiment consisting of 5-8 animals per group.

Figure S7. Immunofluorescence from liver sections. Liver sections were stained for Kupffer cells (KC) using anti-CLEC4F antibody (Alexa Fluor 647). KC are shown in pink (white arrows). Phalloidin 594 (green) and Dapi (blue) were used for ACTIN and nucleus staining, respectively. Different animal models are shown perpendicularly and different treatment groups are shown horizontally. Results shown are from one representative animal per group and one representative experiment. For image acquisition, an Olympus BX61VS slide-scanner equipped with a 20x objective with a numerical aperture of 0.75, and the OlyVIA software were used.

Figure S8. Quantification of liver prussian blue stained area. Area of Prussian blue staining for the (A) iron deficiency anemia (IDA) model, (B) anemia of chronic disease (ACD) model and (C) ACD/IDA of the liver sections shown in figure 7, performed with ImageJ. Details described in methods.

Figure S9. Histology of the spleen stained with prussian blue. 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, 200µm. Results shown are from one representative experiment and one representative animal per group. For image acquisition, an Olympus BX61VS slide-scanner equipped with a 20x objective with a numerical aperture of 0.75, and the OlyVIA software were used.