Methods

Mouse model. Townes sickle breeders were obtained from The Jackson Laboratories (Bar Harbor, ME). Mice were bred using a homozygous male by heterozygous female cross strategy. Genotyping was performed by Transnetyx (Cordova, TN). Mice were maintained in the animal facility at Saint Louis University where they were group housed by gender, maintained on a 12-hour light cycle and given *ad libitum* access to food and chow. Diets containing approximately 20 (TD.110849) and 48 (TD.06442) ppm iron were obtained from Envigo Teklad (Madison, WI). Experiments were performed on 8-10 week old mice that had been weaned and maintained on their respective diets until sacrifice. Both sexes were included in all experiments. All animal studies were performed under Institutional Animal Care and Use Committee approved protocols.

Hematologic and serum parameters. Blood was obtained for all analyses by either cardiac or submandibular puncture. Complete blood counts were performed by Idexx laboratories (Westbrook, ME). Indirect bilirubin was quantified with by Quantichrome bilirubin assay kit manufactured by BioAssay Systems (Hayward, CA). Serum iron and transferrin saturation were assayed per standard protocol (Pointe Scientific, Canton, MI). Serum sVCAM-1 was quantified with a mouse sVCAM-1/CD106 Quantikine ELISA (R&D Systems, Minneapolis, MN).

Oxygen gradient ektacytometry (oxygenscan). Blood was obtained via cardiac or submandibular puncture and collected in a Minicollect K2EDTA tube. Oxygenscan measurements were routinely performed 24 hours after blood collection with blood stored at 4^0C. RBCs in whole blood were quantified using an automated cell counter. 250 x 10^6 cells were mixed with 5 mL Oxy Iso PVP solution (Mechatronics, The Netherlands). Samples were analyzed using an oxygenscan equipped laser optical red cell rotational analyzer (Lorrca; Mechatronics, The Netherlands)[1] per manufacturer's protocol and standard parameters (pO_2 control P-term = 25; pO_2 I-term = 2.8).

Quantitative reverse transcription polymerase chain reaction. Tissues were homogenized in guanidinium thiocyanate (Trizol; Ambion, Carlsbad, CA) and RNA was extracted per manufacturer's instructions. RNA was reverse transcribed with iScript RT supermix (Biorad, Hercules, CA). Resulting cDNA samples were analyzed by qPCR using Taqman Gene expression assay primer/probe sets (Applied Biosystems, Foster City, CA). Thermal cycling was performed with a CFX Connect real-time system (Biorad). Thermal cycling consisted of 40 cycles (95^0C for 15 seconds followed by 60^0C for 1 minute). Data were analyzed by the -ΔCt method [2].

Tissue iron concentrations. Nonheme tissue iron concentrations were measured by the bathophenanthroline method previously described [3]. Results are presented as microgram iron per gram dry weight of tissue.

Statistical analyses. Data were analyzed using GraphPad Prism 8 software (LaJolla, CA). Outliers identified by the Rout method were removed where appropriate. Correlations were analyzed by linear regression and strength determined by Pearson coefficient. Statistical significance was determined by Student *t* test, or analysis of variance (ANOVA) with comparisons across groups performed by Kruskal-Wallis test. Differences were considered statistically significant if $p < 0.05$.

References

1. Rab MAE, van Oirschot BA, Bos J, et al. Rapid and reproducible characterization of sickling during automated deoxygenation in sickle cell disease patients. *Am J Hematol*. 2019;94(5):575-584.
2. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25(4):402-408.
3. Torrance J, Bothwell T. Tissue iron stores. In: Cook J, ed. Methods in hematology. Vol. 1. New York, NY: Churchill Livingstone; 1980:90-115.

Supplementary Figure legends

Supplementary Figure S1. Iron restriction improves hematological parameters in Townes sickle mice. Townes sickle mice weaned onto a 20 ppm iron diet demonstrate A) a decrease, although not statistically significant, in MCH and B) an increase in MCV compared to sickle mice weaned onto a 48 ppm diet. Values from sickle trait mice weaned onto the same diets are shown for reference. For panel A, n = 8-10 HbAS mice and 9-13 HbSS mice per group. For panel B, n = 8-11 HbSA mice and 9-13 HbSS mice per group. ppm, parts per million; data represented as means (SEM).*, $p<0.05$; ***, $p<0.005$; ****, $p<0.001$; ns, non-significant

Supplementary Figure S2. Iron restriction decreases markers of hemolysis and improves tissue oxygenation. (A) Renal erythropoietin expression in Townes sickle cell disease mice (HbSS) and sickle trait mice (HbAS) fed a 20 ppm or 48ppm iron diet. (B) Serum sVCAM-1 concentration in Townes sickle cell disease (HbSS) and sickle trait (HbAS) mice fed a 20 ppm or 48 ppm iron diet. (C) Indirect serum bilirubin concentration in Townes sickle cell disease mice (HbSS) and sickle trait mice (HbAS) fed a 20 ppm or 48ppm iron diet. For panel A n=6-10 mice per group for HbAS and 7-14 mice per group for HbSS mice. For panel B, n = 4 HbAS mice per group and 14-16 HbSS mice per group. For panel C, n=7-8 mice per group for HbAS and 13-15 mice per group for HbSS. Epo, Erythropoietin; ppm, parts per million; data represented as means (SEM). *, $p<0.05$; ***, $p<0.005$, ns, non-significant.

Supplementary Figure S3. Iron restriction mitigates tissue iron loading and decreases hepcidin expression. (A) Kidney iron, (B) splenic iron and (C) hepatic iron concentrations in Townes sickle cell disease mice (HbSS) and sickle trait mice (HbAS) fed a 20 ppm or 48ppm iron diet. (D) Hepatic hepcidin expression in Townes sickle cell disease mice (HbSS) or sickle trait mice (HbAS) fed a 20 ppm or 48ppm iron diet. For panel A, n = 8-9 HbAS mice and 9-11 HbSS mice per group. For panel B, n = 12-18 HbAS mice and 15-23 HbSS mice per group. For panel C, n = 8 HbAS and 9-14 HbSS mice per group. For panel D, n = 10-11 HbAS mice and 11-17 HbSS mice per group. ppm, parts per million; data represented as means (SEM).*, $p<0.05$; ***, $p<0.005$, ns, non-significant.

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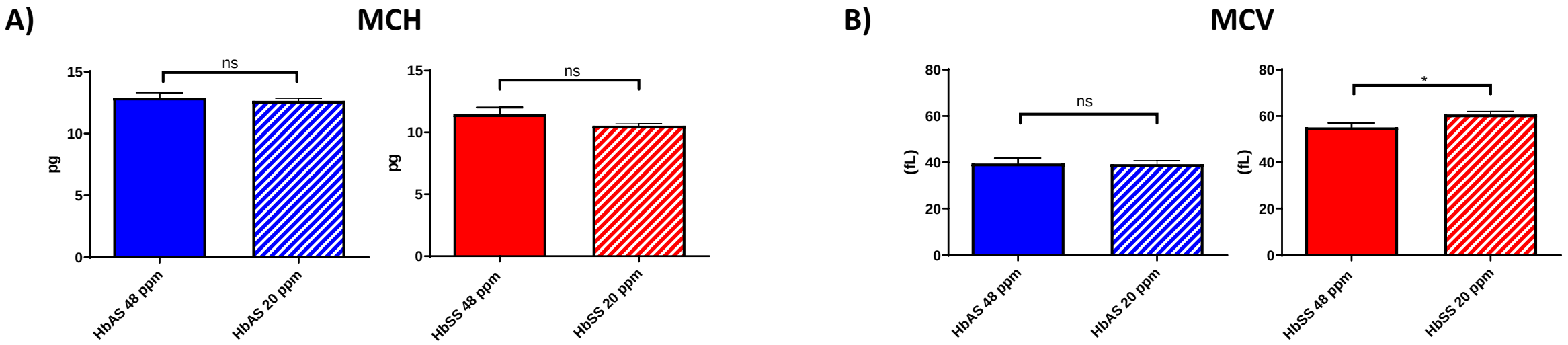

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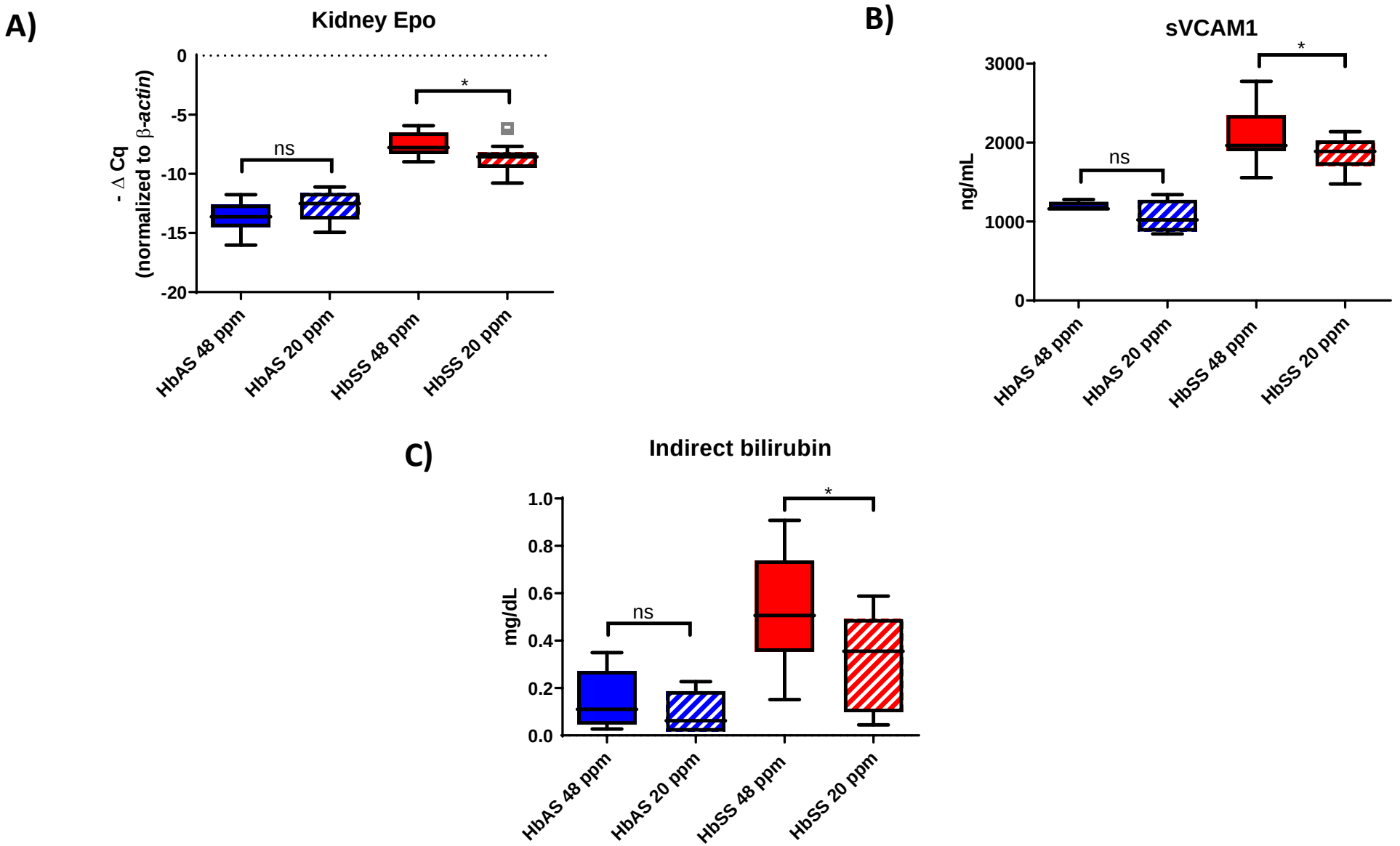

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