Original Research

Systemic Iron Deficiency in a Nonhuman Primate Model of Endometriosis

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Endometriosis is characterized by endometrial tissue development outside the uterus. Anemia and iron depletion do not commonly accompany endometriosis in women, despite chronic abdominal inflammation and heavy menstrual bleeding. The objective of this study was to examine iron kinetics associated with endometriosis by using a NHP model, to better understand the underlying mechanism of abnormal hematogram values in women with endometriosis. Hematologic data from 46 macaques with endometriosis were examined for signs of iron depletion. Bone marrow, liver, and serum were used to elucidate whether iron loss or inflammation best explained the hematologic findings. Additional serum markers and intestinal biopsies from NHP with and without endometriosis were evaluated for patterns in iron kinetics across the menstrual cycle and for relative dietary iron-absorbing capacity. Almost half of the NHP with endometriosis were anemic. Overall, NHP had decreased RBC counts, increased MCV, increased percentage of reticulocytes, decreased serum hepcidin, and decreased hepatic and bone marrow iron. Intestinal expression of ferroportin 1, a mediator of iron absorption, was increased, indicating that despite high dietary iron, intestinal iron absorption did not compensate for iron losses. We concluded that use of oral iron supplementation alone does not replenish iron stores in endometriosis. Consequently, iron stores should be evaluated in women with endometriosis, even without overt clinical signs of anemia.

Abbreviations: α HSP, α -hemoglobin-stabilizing protein

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Endometriosis, characterized by the growth of endometrial tissue implants outside the uterus, affects as many as 200 million women between the ages of 15 and 55 y worldwide.¹⁴ Women with endometriosis can experience severe pelvic and abdominal pain, infertility, and heavy or irregular menses.³⁰ Over time, menstrual losses may result in the depletion of iron stores, with detrimental downstream effects on hematopoiesis and cell metabolism.⁸ Women with endometriosis commonly report fatigue, weakness, and malaise; these are also symptoms of anemia but often are unrecognized as such.²⁵

In addition to excessive blood loss through heavy menses, chronic systemic inflammation is another possible mechanism of iron unavailability in endometriosis. Proinflammatory cytokines produced by endometrial tissue and its associated inflammatory cells may increase serum cytokine concentration and activate the hepcidin-mediated pathway. The peptide hepcidin is produced in the liver and induces the endocytosis of ferroportin 1, an iron exporter. Ferroportin 1 is essential for iron movement throughout the body. The ferroportin-1 transporter in the duodenum catalyzes dietary iron transfer across the basolateral aspect of enterocytes into the circulation. Under inflammatory conditions, upregulation of hepcidin results in diminished expression of ferroportin 1 on the intestinal mucosa basolateral membrane through endocytosis and degradation, reducing

plasma iron absorption.²⁷ Downregulation of the transporter sequesters iron in the liver, bone marrow, or splenic macrophages and decreases overall systemic iron available for cell metabolism and erythropoiesis.¹³

Little is known about iron kinetics and erythropoiesis in women with endometriosis. Studies, based on a few serologic markers of iron homeostasis, report conflicting results.^{18,31} In unpublished preliminary analyses using the Wake Forest Baptist Medical Center electronic record database, we compared preoperative hematologic values of women with ($n = 60$) and without ($n = 30$) surgically confirmed endometriosis. Complete treatment histories and American Society for Reproductive Medicine severity staging for women with endometriosis in the study were unavailable. We found women with endometriosis had significantly decreased Hct ($P = 0.009$, mean $\pm$ SEM = 37.9 ± 0.584) and Hgb ($P = 0.031$, mean $\pm$ SEM = 12.6 ± 0.206) concentrations compared with age-matched controls. We also noted Hct, Hgb, and MCV values below calculated control 95% confidence intervals in the women with endometriosis. Additional, more comprehensive studies in women are essential to verify these initial findings.

The overall goal of the current study was to better understand iron kinetics in endometriosis. Mechanistic studies are essential for developing successful strategies to mitigate endometriosis-associated anemia and other clinical symptoms. Therefore, we examined hematologic parameters and systemic iron status in an established NHP macaque model of spontaneously occurring endometriosis. NHP are more suitable models of endometriosis and iron kinetics than other animal models, because

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Table 1. Hematologic values of cynomolgus macaques with endometriosis

		Mean	SEM	1 SD	95% CI	P
Age, y	Control	18	0.845	5.73	16.6–20.0	0.79
	Endometriosis	18	0.661	4.49	16.7–19.3	—
RBC, $\times 10^6/\mu\text{L}$	Control	6.23	0.121	0.82	6.0–6.5	0.0002 ^a
	Endometriosis	5.40	0.180	1.22	5.0–5.8	—
Hb, g/dL	Control	11.85	0.239	1.62	11.4–12.3	0.31
	Endometriosis	11.41	0.369	2.50	10.7–12.2	—
Hct, %	Control	38.80	0.757	5.14	37.3–40.3	0.19
	Endometriosis	37.07	1.069	7.25	34.9–39.2	—
MCV, fL/cell	Control	62.65	1.026	6.96	60.6–64.7	<0.0001 ^a
	Endometriosis	69.65	1.164	7.90	67.3–72.0	—
MCH, pg	Control	19.12	0.317	2.15	18.5–19.8	0.0003 ^a
	Endometriosis	21.35	0.416	2.82	20.5–22.2	—
MCHC, g/dL	Control	29.32	0.662	4.50	28.0–30.7	0.27
	Endometriosis	30.66	0.309	2.09	30.0–31.3	—
Reticulocytes, % ^b	Control	0.60	0.09	0.29	0.29–0.8	0.006 ^a
	Endometriosis	1.51	0.33	1.09	0.7–2.2	—

All groups contained 46 macaques, except those for reticulocyte count ($n = 11$ each)

^a $P \leq 0.05$

^bValues were back-transformed from $\log(y)$

NHP have physiologically similar menstrual cycles and naturally develop endometriosis (at a maximal rate of 30%).^{4,33} In addition, NHP allow for sampling of tissues (for example, liver and bone marrow) difficult to obtain from healthy women. We hypothesized that iron loss is the primary mechanism underlying symptoms of iron depletion in endometriosis and that the hepcidin-mediated iron sequestration pathway has a secondary role. We also predicted that an optimal window exists during the menstrual cycle when dietary iron absorption would be most efficient to potentially counteract menstrual iron loss.

Materials and Methods

Selection of NHP cohorts. Archived hematologic data from cynomolgus (*Macaca fascicularis*) and rhesus (*M. mulatta*) macaques were collected from databases of clinical records and necropsies performed at the Wake Forest School of Medicine, Clarkson Campus. All animal use was approved by the Wake Forest School of Medicine Animal Care and Use Committee and was performed in accordance with the *Guide for the Care and Use of Laboratory Animals*.³² The Wake Forest School of Medicine is compliant with the Animal Welfare Act and Animal Welfare Regulations and is AAALAC-accredited.^{2,3}

We included samples and data from NHP diagnosed with naturally occurring endometriosis by cyst fluid cytopathology or histopathology (age; mean, 18 y; range, 8 to 32 y; $n = 46$) but no other condition (that is neoplasia, chronic bleeding disorders) or experimental treatment that might alter hematologic parameters. The endometriosis cohort included 40 cynomolgus and 6 rhesus macaques. Samples and data from controls were selected from NHP with a clinically normal uterus and

no histopathologic indications of systemic inflammation noted at necropsy (mean age, 18 y; $n = 46$). In addition, controls had received no experimental treatments that potentially might affect hematologic values. The control cohort consisted of 30 cynomolgus and 16 rhesus macaques. There were no significant hematologic differences between species within each cohort. NHP were housed in either outdoor corals or indoor cages and were fed commercial (Monkey Chow, Fiber-Plus, Teklad, Harlan Laboratories, Indianapolis, IN) or customized diets. Customized diets included variations on an inhouse formulated high saturated fat and high sugar diet. There was no significant difference in dietary iron concentrations between the endometriosis and control groups. Animals had unrestricted access to tap water. Reference intervals for hematologic parameters were calculated by using 95% confidence intervals for data from controls, as established in the literature.¹¹

Evaluation of bone marrow and liver. Bone marrow sections from 12 controls and 10 NHP with endometriosis (a subset of the 46 NHP noted earlier; all samples were from cynomolgus macaques) and were stained for erythrocyte precursors (α -hemoglobin-stabilizing protein [α HSP], rabbit polyclonal antibody, Rockland Antibodies, Limerick, PA; dilution, 1:10,000) and megakaryocytes (CD61, mouse monoclonal antibody, clone 2f2, Thermo Scientific, Waltham, MA; 1:50)²² by using an automated staining system (Leica Bond RX, Leica Biosystems, Buffalo Grove, IL). Epitope Retrieval Solution 2 was used to label α HSP and CD61 at a pH 9 for 20 min at 99 °C; antibody binding was detected by using Fast Red (Polymer Refine Detection Kit DS9390, Leica Biosystems) and DAB (Polymer Refine Detection Kit DS9800, Leica), respectively. Tissues were counterstained with Mayer hematoxylin, and images were digitized by using a slide scanner (NDP Nanozoomer, Hamamatsu Photonics,

Figure 1. RBC counts and indices of RBC regeneration in NHP with endometriosis. (A) RBC counts were decreased ($P = 0.0002$) in NHP with endometriosis ($n = 46$) compared with controls ($n = 46$). Compared with controls, NHP with endometriosis had (B) increased ($P = 0.0003$) MCV (controls, $n = 46$; endometriosis, $n = 46$) and (C) increased ($P = 0.006$) percentage of reticulocytes (controls, $n = 11$; endometriosis, $n = 11$). Data are shown as mean $\pm$ SEM and were analyzed by using Student t tests. *, $P < 0.05$.

Bridgewater, NJ). A total of 3 randomized fields from each digital slide were analyzed by using image analysis software (Image Pro Premiere 9.1, Media Cybernetics, Rockville, MD) to achieve a statistical power of 0.80. A blinded observer evaluated all images; erythroid precursors and megakaryocytes were quantified per square micrometer.

Ten NHP with endometriosis and 10 clinically healthy, age-matched controls from the parent macaque cohort were used for iron analyses. The endometriosis cohort comprised 9 cynomolgus and one rhesus macaque. The control group included 10 cynomolgus macaques. Formalin-fixed, paraffin-embedded liver and bone marrow blocks were cut into 5- μ m (liver) or 3- μ m (bone marrow) sections and stained for iron by using Prussian blue histochemistry. Images were digitized and processed similar to bone marrow, with 3 random images per slide evaluated for blue-channel percentage area by using Image Pro Premiere imaging software (Media Cybernetics). In addition, 20 liver sections ($n = 10$ endometriosis, 10 control) from the overall cohort were directly analyzed for iron concentration by using inductively coupled plasma mass spectrometry (ICP-MS, ARUP Laboratories, Salt Lake City, UT).

Analysis of the hepcidin-mediated iron sequestration pathway. Serum hepcidin (DRG Bioactive Hepcidin 25 High-sensitivity ELISA, DRG International, Springfield Township, NJ) and IL6

(Quantikine HS ELISA, R and D Systems, Minneapolis, MN) were measured by using ELISA kits. Serum iron and total iron-binding capacity were analyzed at Kansas State University through IDEXX Laboratories (Westbrook, ME) by colorimetry on a Cobas Bira biochemical analyzer (Roche Diagnostics, Indianapolis, IN).

Hepcidin and erythroferrone measurement throughout the menstrual cycle. Serum hepcidin (DRG International) and erythroferrone (human FAM132, LifeSpan BioSciences, Seattle, WA) concentrations were evaluated throughout the menstrual cycle by using ELISA kits in 7 clinically healthy, ovulatory cynomolgus macaques (mean age 19 y). Erythroferrone is a peptide released from erythrocyte precursors that supports erythropoiesis in the presence of systemic inflammation (increased serum cytokines) and increased serum hepcidin.¹⁵ These 7 NHP were experimentally naïve, control animals from a previous study.⁷ The NHP received daily vaginal swabs for approximately 10 consecutive weeks to determine the duration and quantity of menses. Serum was collected 3 times each week and measured for progesterone concentration by using a commercially available coated-tube radioimmunoassay (Diagnostic Products, Los Angeles, CA). Macaques were included in this cohort when they had regular menstrual cycles as determined by daily swab data and were otherwise clinically healthy.

Figure 2. Serum hepcidin and free iron in NHP with endometriosis. (A) Serum IL6 concentrations did not differ between controls and NHP with endometriosis ($n = 14$; $P = 0.3$). (B) Hepcidin was decreased ($P = 0.04$) in NHP with endometriosis, ($n = 11$). Serum was also tested for (C) iron (control, $n = 11$; endometriosis, $n = 14$) and (D) total iron-binding capacity (TIBC; control, $n = 11$; endometriosis, $n = 14$). Serum iron was significantly ($P = 0.03$) decreased in the endometriosis group. Data are presented as mean $\pm$ SEM and were analyzed by using Student t tests. Boxes (control) and circles (endometriosis) represent individual data points. *, $P < 0.05$.

Immunoreactivity for ferroportin-1 transporter. Sections of archived duodenum tissue from 7 clinically healthy NHP (all cynomolgus) and 7 NHP with endometriosis (6 cynomolgus, 1 rhesus) were stained for the ferroportin-1 iron transporter (PA5-22993, rabbit polyclonal antibody, Life Technologies, Carlsbad, CA; dilution, 1:600) by using the automated staining system previously described. Vector Red (Vector Laboratories, Burlingame, CA) was used as the chromophore, with Mayer hematoxylin as a counterstain. Positive and negative controls for immunohistochemistry were segments of duodenum from a healthy NHP. Slides were digitized by using a microscope (model BX61VS, Olympus, Waltham, MA) and Pike camera (Olympus). Images were then analyzed for overall intestinal mucosa integral optical density ($\text{lum}/\mu\text{m}^2$) by using Image Pro Premiere 9.1 (Media Cybernetics). Immunofluorescence was used to verify the distribution of ferroportin immunoreactivity: Fast Red (Leica Biosystems) was the fluorochrome, and slides were coverslipped with Prolong Gold Antifade mounting medium with DAPI (Life Technologies). NHP in the endometriosis and control groups were matched by stage of menstrual cycle according to uterine histology and vaginal swab data.²⁹ A total of 7 NHP were studied in the follicular phase ($n = 4$ with endometriosis,

$n = 3$ controls) and 6 in the luteal phase ($n = 3$ with endometriosis, $n = 3$ controls).

Statistical analyses. Power calculations were determined by using G*Power.¹⁰ Statistical analyses were performed by using Prism 7 (GraphPad Software, La Jolla, CA). Two-sided Student t or Mann–Whitney tests were used to compare means between hematology and tissue iron groups, as appropriate, according to rules of normalcy. Differences in hepcidin and erythroferone concentrations across menstrual cycle days were analyzed by using Kruskal–Wallis and Dunn multiple-comparison tests. Two-way ANOVA was used to compare inverse-transformed intestinal ferroportin-1 immunoreactivity data between disease status and phase of the menstrual cycle. A P value of less than 0.05 was considered significant for all statistical methods.

Results

Hematology evaluation. Age (Table 1), body weight (data not shown), and diet composition (data not shown) did not differ significantly between macaques with or without endometriosis. The severity and distribution of endometriosis correlating to American Society for Reproductive Medicine guidelines were

Figure 3. Hepatic and bone marrow iron concentrations in NHP with endometriosis. (A) Bone marrow iron concentrations from NHP with endometriosis were significantly ($P = 0.022$) decreased when assessed by using Prussian blue histochemical staining and histomorphometric analyses (control, $n = 10$; endometriosis, $n = 10$). (B) Representative images of bone marrow stained with Prussian blue; original magnification, 400 $\times$. Iron formed large clumps within the control bone marrow (top, large arrows). (C) Hepatic iron was decreased ($P = 0.013$) in NHP with endometriosis (control, $n = 10$; endometriosis, $n = 9$). (D) Hepatocytes from NHP with and without endometriosis, stained with Prussian blue; original magnification, 400 $\times$. Kupffer cells within sinusoids often contained iron (top, small arrows). (E) Hepatic iron values were validated by using direct inductively coupled plasma-mass spectroscopy ($P = 0.036$; control, $n = 10$; endometriosis, $n = 10$). Data are presented as mean $\pm$ SEM. Boxes (control) and circles (endometriosis) represent individual data points. Data in panels A, C, and E were analyzed by using Student t tests; *, $P < 0.05$.

Figure 4. Erythroid precursors in NHP with endometriosis. Bone marrow was stained for (A) erythrocyte precursors (EP) by using αHSP and for (B) megakaryocytes by using CD61. Neither EP nor megakaryocyte count (no. of cells per square micrometer; $P = 0.998$ and $P = 0.053$) differed between the 2 groups. Data were analyzed by using Student *t* tests and are presented as mean $\pm$ SEM. (C) Representative images of bone marrow EP (Fast Red stain) and megakaryocytes (DAP stain). Original magnification, 20 $\times$.

grade I in 37.5% of NHP with endometriosis, grade II in 12.5% of these animals, and IV in the remaining 50% of NHP with endometriosis.^{1,23} At necropsy, 28% (13 of 46) of NHP with endometriosis had clinical signs of anemia (lethargy, pale mucous membranes). In addition, 41% (19 of 46) of NHP with endometriosis were classified as anemic, according to decreased RBC counts, Hgb concentrations, and Hct values. More than 57% had at least one reported indicator of RBC regeneration, including anisocytosis, polychromasia, poikilocytosis, or reticulocytosis (data not shown). None of the control animals had clinical signs of anemia. Compared with healthy controls, NHP with endometriosis had significantly lower RBC counts (13% decrease, $P = 0.0002$), increased MCV (11% increase, $P < 0.0001$), and increased percentage of reticulocytes (124% increase, $P = 0.006$; Figure 1). Although MCH was increased ($P = 0.0003$) in NHP with endometriosis, this likely was an artifact.

Iron kinetics and storage and hematopoiesis. Despite the apparent histologic presence of abdominal inflammation in NHP with endometriosis, mean serum IL6 did not differ between groups (43% increase, $P = 0.30$; Figure 2 A). In addition, although increased systemic IL6 generally increases hepcidin production from hepatocytes, NHP with endometriosis had significantly lower levels of hepcidin than controls (73% decrease, $P = 0.04$; Figure 2 B).¹³

Serum iron and total iron-binding capacity are short-term markers of iron movement; when interpreted together, they may be used to extrapolate systemic iron status.^{27,28} Serum iron was significantly decreased (23% decrease, $P = 0.03$) in NHP with endometriosis compared with controls (Figure 2 C), but total iron-binding capacity did not differ between groups ($P = 0.36$; Figure 2 D). Hemolysis of samples also may account for differences in serum iron measurement, but scores did not differ between groups (data not shown).

Iron levels in bone marrow were significantly decreased in NHP with endometriosis (55% decrease, $P = 0.02$; Figure 3 A and B). These NHP also had significantly decreased hepatic iron content (histomorphometry: 53% decrease, $P = 0.01$; direct measurement: 63% decrease, $P = 0.04$; Figure 3 C and D). Histomorphologic assessment of liver demonstrated that distribution of iron within hepatocytes compared with Kupffer cells did not differ between NHP with or without endometriosis. A decrease in hepatic iron concentration was confirmed by using mass spectroscopy for direct iron quantification (Figure 3 E). On immunohistochemistry and light microscopy, numbers of erythroid precursors did not differ significantly between groups ($P = 0.99$), but megakaryocytes tended to be increased in those with endometriosis (77% increase, $P = 0.05$; Figure 4), consistent with findings in women with endometriosis.⁹

Figure 5. Hepcidin and erythroferrone concentrations throughout the menstrual cycle in healthy NHP. (A) Hepcidin and erythroferrone concentrations throughout the menstrual cycle in 7 healthy cynomolgus macaques. The sampling captured 2 complete menstrual cycles per NHP (30 samples per animal; 205 samples total). According to ANOVA with multiple-comparison analysis, neither peptide changed in serum concentration (mean $\pm$ SEM) throughout the menstrual cycle. (B) Representative menstrual cycle in a clinically healthy NHP, with serum hepcidin, erythroferrone, and progesterone concentrations depicted across the menstrual cycle. Gray box, menses.

Serum hepcidin and erythroferrone concentrations during the menstrual cycle. Serum concentrations of hepcidin ($P = 0.84$) and erythroferrone ($P = 0.99$) did not differ between macaques with and without endometriosis (Figure 5 A). Similarly, multiple comparisons among samples collected on the same calendar day did not reveal significant differences in erythroferrone or hepcidin concentrations ($P > 0.9999$). Representative menstrual cycles and serum hepcidin, erythroferrone, and progesterone concentrations are shown in Figure 5 B.

Immunoreactivity of duodenal ferroportin-1 transporter. Compared with controls, NHP with endometriosis had increased expression of the ferroportin-1 transporter in the duodenum (220% increase, $P = 0.01$; Figure 6 A). Expression of the ferroportin-1 transporter did not differ between duodenal sections taken from NHP in the follicular compared with luteal phases ($P = 0.54$; Figure 6 B). Distribution of ferroportin was primarily apical and intracellular in both groups of NHP, with stronger immunoreactivity in those with endometriosis (Figure 6 C). Figure 6 D depicts intestinal Prussian blue histochemical iron staining

in NHP with and without endometriosis. In this analysis, control NHP had more iron-laden cells within villi.

Discussion

In the current study, many NHP with endometriosis had RBC indices indicative of anemia. Decreased hepatic and bone marrow iron stores, decreased serum hepcidin, and decreased serum iron levels in these animals supports iron loss as the major mechanism underlying the hematologic abnormalities. In addition, NHP with endometriosis had elevated levels of the ferroportin-1 transporter in duodenal sections and an apparently greater capacity to absorb dietary iron than control macaques. Regardless, NHP with endometriosis had decreased hepatic and bone marrow iron stores, indicating that dietary iron supplementation alone may be insufficient to restore systemic iron homeostasis in cases of endometriosis. Altogether, the mechanistic findings presented here may have broad implications in women with endometriosis, and further studies in women are needed.

Figure 6. Ferroportin-1 transporter immunoreactivity (mean $\pm$ SEM; back-transformed from inverse values) in the small intestine of NHP with endometriosis. (A) Sections of duodenum from NHP with ($n = 6$) and without ($n = 7$) endometriosis were evaluated for ferroportin-1 transporter immunoreactivity. Ferroportin-1 immunoreactivity was significantly ($P = 0.01$, Student t tests) increased in NHP with endometriosis. (B) Data grouped by menstrual cycle phase (control: follicular phase, $n = 4$; luteal phase, $n = 3$; endometriosis: follicular phase, $n = 4$; luteal phase, $n = 2$) showed no difference ($P = 0.54$) in ferroportin-1 immunoreactivity. (C) Representative sections of duodenum from control NHP and NHP with endometriosis stained for ferroportin 1, original magnification, 400 $\times$; +, positive immunohistochemical control; -, negative immunohistochemical control. (D) Prussian blue staining of intestinal villi from NHP with and without (control) endometriosis; original magnification, 200 $\times$. *, $P < 0.05$.

Our NHP model had 2 hematologic parameters that are not classically considered characteristic of systemic iron loss: increased MCV and increased reticulocyte percentage.⁵ The

absence of increased erythroid precursors in bone marrow led us to postulate that systemic iron was low enough to diminish—but not stop—erythropoiesis in most NHP with endometriosis.

In a rodent model of iron kinetics, systemic iron loss through phlebotomy resulted in regenerative anemia when animals consumed an iron-sufficient diet.²⁷ These findings likely explain the signs of regeneration (that is, increased reticulocyte counts, increased MCV) in our NHP model, in which NHP had decreased iron stores and chronically lost blood through menses despite consumption of a high-iron diet.

We anticipated that serum IL6 in our macaques would be increased due to the inflammation associated with endometriosis. However, serum IL6, in fact, was not significantly increased, likely due to the chronicity of endometrial lesions, with some NHP receiving a diagnosis years before evaluation at necropsy. Women with endometriosis have variable serum and peritoneal fluid levels of proinflammatory cytokines, including IL6, but there is a knowledge gap regarding the circulating inflammatory response in NHP with endometriosis.^{16,19,21,24,26} Decreased serum hepcidin levels are likely mediated through transmembrane protease serum 6 by low hepatic iron stores.¹² Low iron increases the transcription of this protease gene to reduce hepcidin production in hepatocytes. Increasing both IL6 and hepcidin in NHP with endometriosis likely would lead to hematologic abnormalities due to iron sequestration in liver and bone marrow. If so, hepatic or bone marrow iron stores would be similar between the 2 groups. However, given the significant intergroup difference, additional studies are needed to determine whether other serum cytokines or proteins (for example, TNF α , C-reactive protein) are involved in endometriosis and iron kinetics independent of the hepcidin-mediated pathway.

Dietary iron supplementation is commonly used to prevent clinical signs of iron deficiency in women. Ferroportin-1 transporters in the duodenum, present on both the basolateral and apical membrane of enterocytes, promote dietary iron absorption and enhance movement of iron throughout the body to areas of need.¹³ In our macaque model, high dietary iron and abundant expression of duodenal ferroportin 1 appeared insufficient to restore serum iron or bone marrow and hepatic iron stores associated with endometriosis. This observation suggests that iron supplementation alone may be inadequate to correct the hematologic defects in NHP. We suspect the same is true for women, but the translational implications are unknown. Antiinflammatory therapies, proposed as therapeutic alternatives to suppression of the hypothalamic–pituitary–ovarian axis for treatment of pelvic pain, also may help to correct iron deficiency associated with endometriosis.³⁰ Future studies with such agents should address this endpoint. In addition, hepcidin and erythroferrone concentrations in clinically healthy NHP did not appear to vary during the menstrual cycle. This result suggests that there may be no optimal time in the cycle for iron supplementation. The increase in ferroportin-1 immunoreactivity is consistent with our finding of decreased serum hepcidin in endometriosis, given that hepcidin concentrations are inversely associated with internalization and degradation of ferroportin 1.²⁰

Several limitations of this study should be taken into account. First, we examined iron homeostasis at a single time point (that is, necropsy) for each NHP. Systemic iron concentrations are constantly in flux, and the mechanism driving iron depletion may change from initial disease development. Therefore, a longitudinal study is needed to better address fluctuations and interactions between inflammatory mediators and systemic iron levels. Second, significant variations in hepcidin or erythroferrone levels are likely to be nullified by the tightly controlled, high dietary iron present in NHP diets, including the one we fed to our NHP.²⁰ Iron in NHP diets is plant-based and less bioavailable than that of meat-based iron.¹⁷ Women potentially consume

more meat-based iron than NHP at variable levels, which complicates the use of ferroportin-1 immunoreactivity as a relative measure of dietary iron absorption. Third, like women, NHP have individual variability in menstrual cycle duration and timing of hormone peaks. Evaluating serum hepcidin and erythroferrone data according to cycle day, regardless of cycle duration, potentially increases variability in the data and decreases the opportunity to detect significant differences. To correct for these differences, we performed regression analyses comparing serum progesterone levels to hepcidin or erythroferrone. However, serum progesterone levels did not correlate with either peptide. Finally, NHP with endometriosis potentially have more exaggerated changes in serum hepcidin and erythroferrone than healthy animals. Increased blood loss during menses in NHP with endometriosis likely would result in sharp decreases in hepcidin production and increases in erythroferrone, which we did not observe in our macaques.^{13,30}

In conclusion, the use of high dietary iron alone did not replenish iron stores in NHP with endometriosis. Iron deficiency and anemia often accompany endometriosis but may be underrecognized. Additional studies are needed to understand systemic and intralesional iron kinetics in this disease more fully.

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