Ferroportin Expression in Adipocytes Does Not Contribute to Iron Homeostasis or Metabolic Responses to a High Calorie Diet

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SUMMARY

The iron exporter, ferroportin, has been proposed to have a key role in adipocyte iron homeostasis. Contrary to previous reports, we show that adipocyte-specific ferroportin deletion in mice does not alter adipocyte iron loading, adipokine expression, or glucose homeostasis.

BACKGROUND & AIMS: Iron has an increasingly recognized role in the regulation of adipose tissue function, including the expression of adipokines involved in the pathogenesis of nonalcoholic fatty liver disease. The cellular iron exporter, ferroportin, has been proposed as being a key determinant of adipocyte iron homeostasis.

METHODS: We studied an adipocyte-specific ferroportin (Fpn1) knockout mouse model, using an Adipoq-Cre recombinase driven Fpn1 deletion and fed mice according to the fast food diet model of nonalcoholic steatohepatitis.

RESULTS: We showed successful selective deletion of Fpn1 in adipocytes, but found that this did not lead to increased adipocyte iron stores as measured by atomic absorption spectroscopy or histologically quantified iron granules after staining with 3,3’-diaminobenzidine–enhanced Perls’ stain. Mice with adipocyte-specific Fpn1 deletion did not show dysregulation of adiponectin, leptin, resistin, or retinol-binding protein-4 expression. Similarly, adipocyte-specific Fpn1 deletion did not affect insulin sensitivity during hyperinsulinenmic–euglycemic clamp studies or lead to histologic evidence of increased liver injury. We have shown, however, that the fast food diet model of nonalcoholic steatohepatitis generates an increase in adipose tissue macrophage infiltration with crown-like structures, as seen in human beings, further validating the utility of this model.

CONCLUSIONS: Ferroportin may not be a key determinant of adipocyte iron homeostasis in this knockout model.
Further studies are needed to determine the mechanisms of iron metabolism in adipocytes and adipose tissue. (Cell Mol Gastroenterol Hepatol 2018;5:319–331; https://doi.org/10.1016/j.jcmgh.2018.01.005)

Keywords: Iron; Ferroportin; Adipose Tissue; Nonalcoholic Fatty Liver Disease.

Nonalcoholic fatty liver disease (NAFLD) affects approximately 1 billion people worldwide. Many of these individuals develop nonalcoholic steatohepatitis (NASH) and hepatic fibrosis, which can lead to liver failure and hepatocellular carcinoma.²⁴ Treatments that effectively alter the natural history of this disease are lacking and a greater understanding of its pathogenesis is essential to develop such therapies. Dysfunctional adipose tissue has been shown to be central to the pathogenesis of insulin resistance and NASH.⁵ Adipose tissue serves as the predominant source of liver fat in NASH and is considered to be a superior model of selective adipocyte ferroportin knockout (FKO), and Adipoq-fl/fl mice were backcrossed for at least 8 generations onto a C57BL/6 background. Male Fpn1/fl/fl mice then were crossed with female heterozygous C57BL/6 Adipoq-Cre¹⁻/⁻ mice expressing Cre recombinase under the control of Adipoq (adiponectin gene) promotor regions on a bacterial artificial chromosome transgene (Jackson Laboratory, Bar Harbor, ME). This generated both Adipoq-Cre:Fpn1/fl/fl, adipocyte-specific ferroportin knockout (FKO), and Fpn1/fl/fl (Flx) littermate control mice.

Methods
Experimental Animals
Mice with loxP fragments inserted in exons 6 and 7 of the mouse ferroportin gene (Fpn1flo/flo mice) on a 129/SvEvTac background were a kind gift from Professor Nancy Andrews (Duke University, Durham, NC). Fpn1flo/flo mice were backcrossed for at least 8 generations onto a C57BL/6 background. Male Fpn1flo/flo mice then were crossed with female heterozygous C57BL/6 Adipoq-Cre¹⁻/⁻ mice expressing Cre recombinase under the control of Adipoq (adiponectin gene) promotor regions on a bacterial artificial chromosome transgene (Jackson Laboratory, Bar Harbor, ME). This generated both Adipoq-Cre:Fpn1flo/flo, adipocyte-specific ferroportin knockout (FKO), and Fpn1flo/flo (Flx) littermate control mice.

After weaning, mice were housed singly. Sixteen-week-old male mice were randomly assigned, using a computerized random allocation sequence generator, to receive either control diet or fast food diet for 25 weeks until the end of the experiment. Control diet mice were provided with drinking water and fast food diet mice were supplied with 42 g/L high-fructose corn syrup (23.1 g/L fructose, 18.9 g/L glucose; Chem-Supply, Gillman, Australia) in the drinking water. Diets were supplied by Specialty Feeds (Glen Forrest, WA, Australia). Mice had ad libitum access to diet and water (control diet) or high-fructose corn syrup in water (fast food diet). The key constituents of the diets are outlined in Table 1.

At 41 weeks of age, mice were weighed. After a 5-hour fast, mice received an intraperitoneal injection of either 0.75 ml/g humulin R insulin (Eli-Lilly, Indianapolis, IN) in sterile 0.9% sodium chloride (0.15 ml/µL; Pfizer, New York), or 5 µL/g 0.9% sodium chloride alone. After 10 minutes, mice were sacrificed as previously described.²⁵ Whole liver and epididymal fat pad weights were recorded. Liver and epididymal fat pad samples were fixed in formalin for histology. Liver samples were snap frozen in...
liquid nitrogen and stored at -80°C. Liver and spleen samples were dried at 110°C for 72 hours for measurement of tissue iron concentration. Blood was collected by cardiac puncture and serum was stored at -80°C. Adipocytes were isolated from epididymal fat pads after collagenase-dispase digestion as previously described and stored at -80°C.

All experiments were performed with approval from the Animal Ethics Committee of the QIMR Berghofer Medical Research Institute and were conducted in accordance with the NHMRC code for the care and use of animals for scientific purposes. Mice were housed in a temperature-controlled environment (23°C) in a 12:12 hour light:dark cycle. All authors had access to the study data and reviewed and approved the final manuscript.

Glucose Tolerance Tests
Glucose tolerance tests were performed 1 week before sacrifice, at 40 weeks of age. After a 5-hour fast, mice were given 1 g/kg glucose via the intraperitoneal route. Tail vein sampling was performed at 0, 15, 30, 60, and 120 minutes and blood glucose was measured using an Accu-Chek Performa II hand-held glucometer (Roche, Basel, Switzerland).

Hyperinsulinemic–Euglycemic Clamp Studies
Hyperinsulinemic–euglycemic clamps were performed as previously described in 6-hour–fasted mice. An initial 2-minute priming dose of insulin (150 mU/kg/min) was followed by constant infusion at a rate of 15 mU/kg/min. Maintenance of euglycemia was achieved by a variable infusion of 25% glucose solution. Steele’s steady-state equation was used to calculate glucose turnover.

Tissue Iron Concentration
Hepatic and splenic iron concentrations were measured as previously described. Adipocyte iron concentration was performed on isolated adipocytes by atomic absorption spectroscopy (AAS). Approximately 100 mg of adipocytes for each animal were weighed and then dried at 60°C for 60 hours and 100 µL concentrated nitric acid was added. Samples then were incubated at 60°C for 30 minutes, before dilution 1:5 with zero standard (0.2% nitric acid). Standards over a range of 0–25 µmol/L were prepared using iron pure single element standard 1000 mg/L iron in 2% nitric acid (Perkin Elmer, Waltham, MA). All samples (including standards, quality control, and analytical samples) were diluted further 1:3 with 10 g/L palladium matrix modifier for graphite furnace AAS (Merck Millipore, Darmstadt, Germany). AAS was performed at a wavelength of 372 nm using an AA280Z Zeeman Atomic Absorption Spectrometer.

Table 1. Major Components of Experimental Diets

| Dietary component               | Control diet | Fast food diet |
|---------------------------------|--------------|----------------|
| Protein, % weight              | 13.6         | 17.4           |
| Total fat, % weight            | 4.0          | 20             |
| Total digestible carbohydrate, % weight | 64.8         | 48.2           |
| Digestible energy, MJ/kg       | 15.1         | 18.6           |
| Cholesterol, % weight          | 0            | 0.15           |
| Casein (acid), g/kg            | 140          | 180            |
| Sucrose, g/kg                  | 100          | 341            |
| Clarified butter (ghee), g/kg  | 0            | 200            |
| Wheat starch, g/kg             | 472          | 82             |
| Dextrinized starch, g/kg       | 155          | 0              |
| Iron, mg/kg                    | 75           | 75             |
| High-fructose corn syrup in drinking water, g/L | 0          | 42             |

Glucose Tolerance Tests

| Table 2. Quantitative Reverse-Transcription PCR Primer Sequences (5' to 3') |
|------------------|------------------|
| Forward primer   | Reverse primer   |
| Tr1              | GAGGCAGACCTTGAGCTCTT | TGACTGAGATGGCCGAGAAC |
| Fpn1             | GCCACTGCCATCACAATCC | TGGAGTTCTGCACACCATTGAT |
| Hamp1            | TTGCAGATACCAGTCGAGAAAG | GGAATGGCTGCTAGCTAGTTG |
| Adiponectin      | GGGATGTCAGTCTCTTG | TCCAGGCTTCCTTTCTGT |
| Leptin           | GCAGTGCTATTCGAGAAACCC | GGAATGAAATCCAAGCCAGTGAC |
| Resistin         | CATGCCACTGTGTCGCACATCGAT | ACTCCCTCTGGAGAGACTGT |
| Rbp-4            | TGTAGCCTCTCTTCCAGCGA | ACGAGTGCCATCCAGATGCTTG |
| B2-mg            | CTGATACATACTGCTACGAGTATTA | ATGAATCTTACGAGATCATGAT |
| Btf-3            | TGGCAGCAAAACACCTCACCC | AGCTTACGCAACTTTCAAC |
| Gapdh            | TCTGGCTACACGCCACCACTTGCTAG | GGCCTGCTCCACACCTTTGAT |
| Pol2a            | AGCTCTGCTCTGATTCCGG | CTGATCTGCTGTGATCCCTGC |
| β-actin          | CATTCGCTGACAGTACGAAAGG | TGCTGGAAGGTCGAGATGAGG |
| Hprt             | GAGCTGATTGAGACAGGAGA | GAGGGCCACATGGTAGTGA |
| Hmox1            | CACTTGGAGATGACACCTGAG | GTGTTTCCCTGTCAGCATACC |

Tr1, basic transcription factor-3 gene; B2-mg, β2-microglobulin gene; Gapdh, glyceraldehyde-3-phosphate dehydrogenase gene; Hmox1, heme oxygenase 1; Hprt, hypoxanthine guanine phosphoribosyl transferase gene; Pol2a, RNA polymerase II subunit A gene.
(Varian, Palo Alto, CA) with a GTA 120 Graphite Tube Atomizer (Agilent Technologies, Santa Clara, CA). A Zeeman background correction was used. The final results were expressed per gram wet weight.

**RNA Extraction, Real Time Quantitative Polymerase Chain Reaction, and DNA Electrophoresis**

RNA was extracted from liver and adipocyte homogenates using Trisure reagent (Bioline, London, UK). Samples were treated with DNase 1 (Invitrogen, Carlsbad, CA) and complementary DNA (cDNA) was synthesized from 1 μg RNA (liver) and 500 ng RNA (adipocytes) using a Sensifast cDNA synthesis kit (Bioline). For quantitative reverse-transcription polymerase chain reaction (PCR), a ViiA7 real-time PCR machine (Invitrogen) with a Sensifast cDNA synthesis kit (Bioline). Samples underwent thermal cycling as follows: 95°C for 2 minutes, 40 cycles at 95°C for 5 seconds, followed by 63°C for 20 seconds before a melt-curve analysis. Relative mRNA expression was determined by calibration of cycle threshold values to the standard curve of pooled cDNA samples and normalized to the geometric mean of 3 reference genes (basic transcription factor-3, glyceraldehyde-3-phosphate dehydrogenase, β-2-microglobulin for liver samples, and RNA Polymerase II Subunit A, β-actin, and hypoxanthine guanine phosphoribosyl transferase for adipocyte samples). Primer sequences are provided in Table 2.

For confirmation of adipocyte-specific ferroportin knockout, 10 μL of adipocyte *Fpn1* DNA amplification product, created using primers flanking exons 6 and 7 (Table 2) (thermal cycling: 95°C for 2 minutes, 40 cycles at 95°C for 5 seconds, followed by 63°C for 45 seconds) was mixed with 2 μL of 6× DNA loading buffer (New England Biolabs, Ipswich, MA). Samples were electrophoresed at 110 V for 40 minutes in a 1.5% agarose gel (Bioline) mixed with a Sensifast cDNA synthesis kit (Bioline). For quantitative reverse-transcription polymerase chain reaction (PCR), a ViiA7 real-time PCR machine (Invitrogen) with a Sensifast cDNA synthesis kit (Bioline). Samples underwent thermal cycling as follows: 95°C for 2 minutes, 40 cycles at 95°C for 5 seconds, followed by 63°C for 20 seconds before a melt-curve analysis. Relative mRNA expression was determined by calibration of cycle threshold values to the standard curve of pooled cDNA samples and normalized to the geometric mean of 3 reference genes (basic transcription factor-3, glyceraldehyde-3-phosphate dehydrogenase, and β-2-microglobulin for liver samples, and RNA Polymerase II Subunit A, β-actin, and hypoxanthine guanine phosphoribosyl transferase for adipocyte samples). Primer sequences are provided in Table 2.

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**Figure 1. Tissue and body weights.** (A) Total body weight. Total body weight was increased in animals fed the fast food diet (*P < .0001*), but genotype effect was not significant (NS, 2-way ANOVA). (B) Liver weight. Liver weight was increased in animals fed the fast food diet (*P < .0001*), but the gene effect was NS (2-way ANOVA). (C) EFP weight. There was a significant interaction between diet and genotype effects (*P = .03*, 2-way ANOVA). Post hoc analysis showed lower EFP weight in FKO mice compared with Flox mice fed the fast food diet (*P = .02*), and increased weight with the fast food diet for both Flox and FKO mice (*P = .0001* and *P < .0001* respectively, Sidak’s multiple comparisons test); n = 8–12 per group.

**Immunoblotting**

A total of 8 μL of 1:1000 mouse serum was electrophoresed on 2% Metaphor Agarose gels (Lonzza, Basel, Switzerland) for 75 minutes at 75 V. Protein was transferred onto polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA) over 60 minutes at 100 V. Blocking was performed using 5% skim milk powder. A 1:10,000 dilution of primary antibody against adiponectin (MAB3608; Merck Millipore) was applied to the membranes. A 1:50,000 dilution of goat anti-mouse horseradish peroxidase antibody (Invitrogen) was applied as a secondary antibody. Visualization was performed using a Supersignal West Femto chemiluminescent kit (Thermo Fisher Scientific, Waltham, MA) on an ImageQuant LAS 500 machine (GE Healthcare Life Sciences).

**Histologic Assessment and Hepatic Hydroxyproline Assays**

Formalin-fixed samples of liver and epididymal fat pad (EFP) were embedded in paraffin. Liver sections were stained with H&E for assessment of steatohepatitis and Sirius Red for assessment of fibrosis. Scoring was performed according to criteria established by Kleiner et al.27 Further liver sections were stained with Oil Red O and the percentage area stained was measured using ImageJ software, version 2 (National Institutes of Health, Bethesda, MD). EFP sections were stained with H&E and the absolute count of macrophage clusters over 10 high-power fields (×400) was determined. Additional EFP sections were stained with 3,3′-diaminobenzidine-enhanced Perls’ stain and eosin counterstain. The average count of iron granules within adipocytes in 5 adjacent high-power fields (×400 magnification) was determined. Small iron granules were counted individually (score, 1), granules filling the whole cell scored 10. All histologic assessments were performed by an expert histopathologist blinded to the study group. Photomicrographs were created using a MicroPublisher 3.3 RTV camera (Q Imaging, Surrey, Canada) and a Biological System Microscope CX41.
A hepatic hydroxyproline assay was performed as previously described.28

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software, version 7.03 (GraphPad, San Diego, CA). Hyperinsulinemic–euglycemic clamp data and glucose concentrations during glucose tolerance tests were analyzed using a 2-tailed Student t tests. For liver histology scoring, Mann–Whitney tests were used to compare genotypes for each diet. For all remaining data, 2-way analysis of variance (ANOVA) was used to assess the effect of diet and genotype. If a significant interaction ($P < .05$) was found, Sidak’s multiple comparison test was used to compare between genotypes for each diet and between diets for each genotype. In cases in which no interaction was found, $P$ values for the diet and genotype effect are indicated. Data presented on box and whisker plots show bars representing the median and interquartile range, with whiskers representing the maximum and minimum values.

Results

Fast Food Diet Was Associated With Increased Body, Liver, and Epididymal Fat Pad Weights

Initial body weight was comparable across all 4 groups (all nonsignificant, Sidak’s multiple comparisons test, data not shown). Consistent with the description of the fast food diet model,22 mice fed with the fast food diet had greater final body weight ($P < .0001$) and liver weight ($P < .0001$, 2-way ANOVA) (Figure 1). EFP weight was higher in both genotypes with the fast food diet ($P = .0001$ Flox mice, $P < .0001$ FKO mice, Sidak’s multiple comparisons test) (Figure 1).

Figure 2. Adipocyte-specific ferroportin knockout does not alter adipocyte iron phenotype. (A) Representative DNA electrophoresis blot of adipocyte Fpn1 reverse-transcription PCR products showing the predicted 163-bp band in adipocytes from FKO but not Flox mice. (B) TIR1 mRNA expression. Diet and genotype effects were both NS (2-way ANOVA; $n = 8–12$ per group). (C) Adipocyte iron concentration. Diet and genotype effects were both NS (2-way ANOVA) ($n = 8–12$ per group). (D) Quantified adipocyte iron granules. Mean count of iron granules in 5 adjacent high-power fields. Original magnification: $\times 400$. Diet and genotype effects were both NS (2-way ANOVA; $n = 8–12$ per group). (E–H) Perls’ staining of epididymal fat pads. Representative light microscopy sections are shown of eosin and 3,3’-diaminobenzidine–enhanced Perls’ stained sections of epididymal fat pads, with arrows indicating small iron granules. Original magnification: $\times 400$. (E) Flox control diet. (F) Flox fast food diet. (G) FKO control diet. (H) FKO fast food diet ($n = 7–12$ per group).
FKO Mice Show Successful Selective Adipocyte Knockout of Ferroportin in Adipocytes, but Not in Other Tissues

Fpn1 primers targeting sequences in exons 5 and 8, which flank the Fpn1 loxP sites, predicted amplification products of 1048 base pairs (bp) for the intact gene and 163 bp for the Cre-recombinase-deleted Fpn1 gene. DNA electrophoresis of Fpn1 quantitative reverse-transcription PCR products from isolated adipocytes showed a clear 163-bp band in all 20 samples of FKO mice and no 163-bp band in all 24 samples in Flox mice, indicating adipocyte Fpn1 deletion in FKO, but not in Flox, mice. A representative gel is shown in Figure 2A. In all liver samples, Flox (n = 9) and FKO (n = 8), and all spleen samples, Flox (n = 7) and FKO (n = 14), the 163-bp band was absent whereas a 1048-bp band was present, indicating a lack of Cre recombinase effect in liver and spleen, irrespective of genotype.

Adipocyte-Specific Ferroportin Deletion Does Not Alter Adipocyte Iron Phenotype

All 3 measures of adipocyte iron loading in adipocytes consistently showed no effect of Fpn1 deletion on iron phenotype (Figure 2B–H). Quantification of Tfr1 mRNA as an inversely related surrogate for cellular iron concentration found no genotype effect (NS, 2-way ANOVA) (Figure 2B). Similarly, adipocyte iron concentration by atomic absorption spectroscopy was not altered by Fpn1 deletion (NS, 2-way ANOVA) (Figure 2C). Histologic assessment of adipocyte iron granules using 3,3’-diaminobenzidine–enhanced Perls’ stain found that iron granule numbers were not increased in FKO mice (NS, 2-way ANOVA) (Figure 2D–H).

Fast Food Diet Leads to Reduced Tissue Iron Concentrations via a Hepcidin-Independent Mechanism

The hepatic iron concentration (HIC) was reduced by fast food diet in FKO mice (P < .0001, Sidak’s multiple comparison test) (Figure 3A). Reduced HIC with the fast food diet does not appear to be explained by an increase in hepcidin (Hamp1) mRNA because Hamp1 mRNA levels were substantially reduced in fast food diet mice in both genotypes (P < .0001, 2-way ANOVA) (Figure 3B). Because HIC is an established regulator of hepcidin transcription,8,29 we normalized Hamp1 mRNA to HIC and found markedly reduced Hamp1/HIC ratios with the fast food diet, likely indicating an appropriate compensatory Hamp1 response to reduced HIC (P < .0001, 2-way ANOVA) (Figure 3C). Splenic
iron concentration also was reduced by fast food diet ($P < .0001$, 2-way ANOVA) (Figure 3D).

**The Fast Food Diet Model Is Associated With Adipose Tissue Macrophage Accumulation**

Clusters of macrophages, resembling crown-like structures (as indicated by arrows in Figure 4C and E) frequently were observed in adipose tissue sections from fast food diet–fed mice, but not in their control diet counterparts ($P < .0001$, 2-way ANOVA). Fpn1 deletion had no effect on the numbers of macrophage clusters (NS, 2-way ANOVA) (Figure 4).

**Adipocyte Expression Is Unchanged in FKO Mice**

There was no effect of Fpn1 deletion on mRNA quantities of the 4 studied adipokines (adiponectin, leptin, resistin, and RBP-4) in both non–insulin-stimulated (basal, fasted state) animals (Figure 5A–D) and in insulin-stimulated animals (Figure 6A–D) (all NS, 2-way ANOVA). Fast food diet led to increased leptin mRNA quantities in both basal-state and insulin-stimulated animals ($P = .03$ and $P = .01$, respectively, 2-way ANOVA). Fast food diet was associated with reduced adiponectin and resistin mRNA in insulin-stimulated animals ($P = .0003$ and $P = .01$, respectively, 2-way ANOVA). A reduction in RBP-4 mRNA was seen in basal-state animals ($P = .01$, 2-way ANOVA) and insulin-stimulated FKO animals ($P = .0008$, Sidak's multiple comparisons test). Total serum adiponectin was unaffected by diet or genotype in basal-state and insulin-stimulated animals (NS in all cases, 2-way ANOVA) (Figure 5E and F). There were also no significant differences with diet or genotype in basal-state or insulin-stimulated animals for high-molecular-weight adiponectin or high-molecular-weight/total adiponectin ratios (all NS, 2-way ANOVA, data not shown).

**Adipocyte-Specific Ferroportin Deletion Does Not Influence Glucose Homeostasis**

Intraperitoneal glucose tolerance tests found no significant differences in blood glucose concentrations for both diets at every time point except 120 minutes (Figure 7A and B). For control diet–fed Flox mice, the mean glucose level at 120 minutes was 16.1 vs 12.8 mmol/L in FKO mice ($P = .02$, Student t test). In fast food diet–fed Flox mice, the mean glucose level at 120 minutes was 13.8 vs 11.3 mmol/L in FKO mice ($P = .046$, Student t test) (Figure 7A and B). The area under the curve (AUC) was measured above the minimum glucose value of 7.7 mmol/L as a baseline. Both diet and genotype effects for AUC were nonsignificant (2-way ANOVA, data not shown). For hyperinsulinemic–euglycemic clamp studies performed on fast food diet–fed mice, the mean body weight between the 2 groups was comparable 42.8 g (Flox) vs 43.4 g (FKO) (NS, Student t test) (Figure 7C). Flox mice had similar basal plasma glucose levels to FKO mice (9.3 vs 9.9 mmol/L) and clamp glucose levels (8.3 vs 8.4 mmol/L) (both NS,
Both groups of mice had substantial increases in mean plasma insulin levels during the clamp studies compared with basal levels (8.3-fold increase in Flox mice [15.7 vs 1.9 ng/mL] and 8.5-fold increase in FKO mice [19.8 vs 2.3 ng/mL]; both $P < .0001$, Student t test, data not shown). Overall, there was no evidence of an effect of FKO on glucose homeostasis. The mean glucose infusion rate was 122.9 $\mu$mol/min/kg in Flox mice vs 121.7 $\mu$mol/min/kg in FKO mice (NS, Student t test) (Figure 7D). The mean rate of whole-body glucose disappearance was 202.5 $\mu$mol/min/kg in Flox mice vs 198.8 $\mu$mol/min/kg in FKO mice (NS, Student t test) (Figure 7F).

Under clamp conditions of hyperinsulinemia, both groups, to a similar extent, failed to suppress endogenous glucose production, a measure of hepatic gluconeogenesis, 79.6 $\mu$mol/min/kg in Flox mice vs 77.2 $\mu$mol/min/kg in FKO mice (NS, Student t test) (Figure 7F).

### Conclusions

In this study, we have shown effective adipocyte-specific ferroportin deletion using an Adipoq-Cre recombinase model. Our study shows 3 key findings. First, ferroportin deletion did not result in any alteration of adipocyte iron phenotype, glucose homeostasis, adipokine regulation, or liver injury. Second, we have shown that the fast food diet is associated with reduced hepatic and splenic iron concentrations with a compensatory hepcidin response. Third, we...
confirm the fast food diet model's utility as a model for NASH and have identified adipose tissue macrophage infiltration, which further validates this model.

We did not find an adipocyte iron loading phenotype despite successful Fpn1 deletion in FKO mice. There are several possible explanations for this. First, ferroportin may not have a significant role in adipocyte iron homeostasis. It is not known whether iron importers such as the divalent metal transporter-1 or an unidentified alternative export mechanism may have more important roles in the regulation of iron content in adipocytes. Second, it is possible that the FKO mice would require a longer period of dietary iron loading to generate such a phenotype even in the absence of adipocyte ferroportin. As such, it may be difficult to determine the importance of adipocyte ferroportin to human beings who may accumulate iron over many years.

Gabrielsen et al\textsuperscript{9} used the AP2-Cre Fpn\textsuperscript{fl/fl} model and reported an iron loading phenotype on the basis of reduced adipocyte Tfr1 mRNA leading to reduced adiponectin transcription and insulin resistance. However, data regarding direct iron assay or histologic assessment of iron were not presented. Tfr1 mRNA quantity is expected to be reduced in iron-loaded cells owing to a negative feedback mechanism involving iron-responsive elements in the Tfr1 gene 3' untranslated region.\textsuperscript{10} However Tfr1 mRNA is a surrogate that is not well validated as a measure of iron loading and particularly not in adipocytes.

The disparity between the study by Gabrielsen et al\textsuperscript{9} and ours could relate to the difference in Cre recombinase site. In addition to its expression in adipocytes, the AP2-Cre has been reported to have some degree of expression in macrophages.\textsuperscript{19–21} Although altered Fpn1 mRNA quantities were not seen in splenic extracts by Gabrielsen et al,\textsuperscript{9} the Adipoq-Cre has been regarded as a more specific Cre recombinase for adipocytes.\textsuperscript{21} Differences between the 2 studies also may relate to a difference in mouse strain. The strain was reported as either "129/SvEvTac or C57BL6" by Gabrielsen et al.\textsuperscript{9} Regardless, it appears that if Cre-lox models of adipocyte ferroportin deletion are to be used as models of adipocyte iron loading, then the iron-loading phenotype needs to be shown more clearly.

Glucose homeostasis was assessed in this study using intraperitoneal glucose tolerance tests and hyperinsulinemic–euglycemic clamp studies. FKO mice had lower blood glucose concentrations at the 120-minute time point, but not at other time points or on the AUC analysis, suggesting that the significance of this result in isolation is doubtful, particularly given the lack of observed change in iron phenotype. Glucose infusion rate and other measures in the clamp studies found no difference between genotypes,
indicating that adipocyte-specific ferroportin knockout does not affect insulin resistance in this model.

We have shown reduced hepatic and splenic iron concentrations as a result of the fast food diet, suggesting reduced body iron stores as a consequence of a high-calorie diet. The hormone hepcidin is considered the key regulator of body iron homeostasis. Hepcidin production is reported to be increased in individuals with NAFLD. It therefore might be expected that low HIC in these mice could be explained by increased Hamp1 expression leading to reduced intestinal iron absorption after the internalization of enterocyte ferroportin. However, Hamp1 expression was decreased markedly in these mice, suggesting appropriate Hamp1 response to reduced hepatic iron stores and is consistent with previous studies. These findings are also in keeping with the established association between iron deficiency and obesity in human beings. Orr et al showed that a high-fat diet led to iron repartitioning with a reduction in HIC and an increase in adipocyte iron concentration via an unknown mechanism, although this was not seen in our model.

The fast food diet model involves 5 months of exposure to a high-calorie diet and high-fructose corn syrup in drinking water in mice housed singly to mimic a sedentary lifestyle. We have found this to be a reliable model for the generation of a phenotype of steatohepatitis and hepatic fibrosis as shown by expert histologic assessment and supported further by quantification of Oil Red O, heme oxygenase 1 mRNA, and hepatic hydroxyproline.

| Table 3. Increased Liver Injury With Fast Food Diet, but Not With Fpn1 Deletion |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                | Control diet    | Fast food diet  |                     |                     |
|                                | Flox            | FKO             | Flox              | FKO              |
| NAS (0–8)                      | 4 (0–6)         | 2 (0–5)         | NS                | 6 (5–7)          | 6 (5–7)         | NS              |
| Steatosis (0–3)                | 2.5 (0–3)       | 1.5 (0–3)       | 3 (3–3)           | 3 (3–3)          | NS              |                     |
| Lobular inflammation (0–3)    | 0 (0–1)         | 0 (0–1)         | 3 (0–2)           | 1 (0–2)          | NS              |                     |
| Ballooning (0–2)               | 1.5 (0–2)       | 0.5 (0–2)       | 2 (2–2)           | 2 (2–2)          | NS              |                     |
| Fibrosis (0–4)                 | 0 (0–1)         | 0 (0–2)         | 2 (1–2)           | 2 (2–2)          | NS              |                     |

NOTE. Median score (range) for NAS (score, 0–8), macrovesicular steatosis (grade, 0–3), lobular inflammation (grade, 0–3), ballooning (grade, 0–2), fibrosis (grade, 0–4). P value was the result of Mann–Whitney tests comparing genotypes for each diet (n = 8–12 per group).

NAS, NAFLD activity score.
increase in adipose tissue macrophage infiltration with crown-like structures. This further validates the applicability of the fast food diet model for use in the study of NASH. We had considered that an increase in adipocyte iron in FKO mice might create an inflammatory state induced by oxidative stress and lead to macrophage infiltration. However, given the observed lack of effect of FKO on iron phenotype, it seems unsurprising that genotype did not affect macrophage infiltration in this model. In our study, we found that EFP weight was lower in FKO mice, although this was seen only in animals fed the control and not the fast food diet. In the context of unaltered iron phenotype and

Figure 8. Fast food diet, but not genotype, leads to steatohepatitis. (A–D) Light microscopy of representative liver sections stained with H&E. Original magnification: ×200. (A) Flox control diet. (B) Flox fast food diet. (C) FKO control diet. (D) FKO fast food diet. (E) Percentage area of liver sections stained with Oil Red O. Oil Red O staining was increased by the fast food diet (P = .0001), but unaltered by genotype (NS, both 2-way ANOVA). (F) Liver heme oxygenase 1 (Hmox1) mRNA. The fast food diet led to increased Hmox1 mRNA (P < .001), but there was no genotype effect (NS, 2-way ANOVA). (G) Hepatic hydroxyproline. Hydroxyproline was increased by the fast food diet (P < .001), but was unaffected by genotype (NS, 2-way ANOVA) (n = 8–12 per group).
lack of effect in fast food diet–fed mice, the significance of this result remains uncertain. When we studied adipokine expression in our model, we found increased adipocyte leptin mRNA and decreased RBP-4 mRNA with a fast food diet. These findings are broadly consistent with human studies of NASH, although conflicting reports exist in the literature.7,40,41

In summary, our findings indicate that the physiological role of ferroportin in adipocytes may be limited and other factors involved in iron homeostasis may be more important in these cells. Because adipocyte iron appears to play a key role in physiological processes, such as appetite regulation, and pathophysiological processes, such as NAFLD and diabetes, a greater understanding of iron metabolism in these cells is clearly a target for future studies.

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