Iron overload results in hepatic oxidative stress, immune cell activation and hepatocellular ballooning injury leading to nonalcoholic steatohepatitis in genetically obese mice.

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**Short title:** Iron induces nonalcoholic steatohepatitis in genetically obese mice

**Key words:** iron excess, hepatocellular ballooning, inflammasome, immune cell activation, reticuloendothelial system

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List of abbreviations: nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), reticuloendothelial system (RES), reactive oxygen species (ROS), malonyldialdehyde (MDA)

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**Abstract:**

The aim of this study was to determine the effect of iron overload in the development of nonalcoholic steatohepatitis (NASH) in a genetically obese mouse model (lepr$^{db/db}$). Leptin-receptor deficient mice were fed a normal or an iron-supplemented chow for 8 weeks and switched to normal chow for 8 weeks. All dietary iron (DI)-fed mice developed hepatic iron overload predominantly in the reticuloendothelial system (RES). Hepatocellular ballooning injury was observed in the livers of 85% of DI mice, relative to 20% of chow-fed lepr$^{db/db}$. Hepatic malonyldialdehyde levels and mRNA levels of antioxidant genes ($Nrf2$, $Gpx1$ and $Hmox1$) were significantly increased in the DI mice. Hepatic mRNA levels of mitochondrial biogenesis regulators $Pgc1\alpha$, $Tfam$, $Cox4$ and $Nrf1$ were diminished in the DI mice. In addition, gene expression levels of cytokines ($Il6$, $Tnf\alpha$) and several innate and adaptive immune cell markers such as $Tlr4$, $Inos$, $CD11c$, $CD4$, $CD8$ and $Ifn\gamma$ were significantly increased in livers of the DI group. Strikingly, $Nlrp3$, a component of the inflammasome and $Il18$, a cytokine elicited by inflammasome activation, were significantly upregulated in the livers of DI mice. In addition, RAW264.7 macrophages loaded with exogenous iron showed significantly higher levels of inflammatory markers ($Inos$, $Tnfa$, $Mcp1$, $Tlr4$). Thus, dietary iron excess leads to hepatic oxidative stress, inflammasome activation, induction of inflammatory and immune mediators, hepatocellular ballooning injury and therefore NASH in this model. Taken together, these studies indicate a multifactorial role for iron overload in the pathogenesis of NASH in the setting of obesity and metabolic syndrome.

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Introduction:

Nonalcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease in the world, and is strongly associated with obesity and the attendant metabolic syndrome (17). NAFLD encompasses a broad spectrum of liver disorders, ranging from simple steatosis to its more severe form, nonalcoholic steatohepatitis (NASH), which progresses to cirrhosis or hepatocellular carcinoma in 15-25% of the patients (17). Oxidative stress is believed to be an important contributor to the pathogenesis of NASH (7, 6, 25). Reactive oxygen species (ROS) initiate an oxidative stress cascade causing lipid peroxidation which damages mitochondria, causing cellular and membranous injury and cell death (7, 6, 25). Iron is known for catalyzing the production of ROS through Fenton’s reaction (7, 6, 25).

In a large human study utilizing 849 patients, we reported that more than 34% of US patients enrolled in the nonalcoholic steatohepatitis clinical research network (NASH CRN) had stainable hepatic iron in liver biopsies (26). We showed in a separate study that elevated levels of serum ferritin, a cytokine associated with iron overload, is an independent predictor of histologic severity and advanced fibrosis among patients with NAFLD (14). In particular, iron in reticuloendothelial system cells [(RES), consisting predominantly of cell types such as Kupffer cells, recruited hepatic macrophages and other immune cells] was associated with advanced disease, including increased steatosis, portal and lobular inflammation and advanced fibrosis in patients with NAFLD (26). In yet another study, we demonstrated that iron deposition in RES compartment
was significantly associated with increased levels of oxidative stress and apoptosis in patients with NAFLD (20).

However, despite the strong association between hepatic RES iron deposition and the worsening of NAFLD, it remains unclear as to whether iron deposition in hepatic RES cells is a cause or consequence of a more severe NAFLD. Given the prevalence of increased iron stores in patients with NASH (25, 26, 14, 20), the supplementation of iron in foods, and the safety and efficacy of reducing iron overload via diet or iron depletion (7, 6, 25), understanding the relationship between hepatic iron loading and NASH carries high significance and broad clinical impact.

In this study, we examined the effect of dietary iron overload in the context of genetic obesity, type 2 diabetes and accompanying metabolic syndrome in a previously described murine model which was shown to recapitulate many of the features of human NASH (11). The goal of the current study was to test the hypothesis that dietary iron overload would lead to a severe NASH phenotype in an obese, diabetogenic mouse model, to characterize the underlying mechanisms in vivo and to confirm these observations in vitro in murine macrophage cells.

**Materials and Methods:**

**Animals and Diet:**

Five-week-old male B6.BKS(D)-*Lepr*\(^{db}\)/J diabetic mice were obtained from The Jackson Laboratory (Bar Harbor, ME). *Lepr*\(^{db/db}\) mice were maintained under standardized conditions of temperature (68-72 °F) and humidity (30-70%), with light from 0600 to 1800 h. At 6-8 weeks of age, mice were fed a normal chow diet (NC, iron content:
280ppm) or a chow supplemented with 2% carbonyl iron (iron content: 20,000 ppm, Test Diets, St. Louis, MO) ad libitum for 8 weeks. After the 8 weeks of iron loading, the mice were allowed to feed the standard rodent chow for an additional 8 weeks. The body weights of all mice were determined weekly. At the study endpoint, fasting mice were sacrificed, and serum and plasma samples were collected by cardiac puncture for assays of glucose, iron parameters and liver enzymes. Livers were rapidly dissected and pieces of the left and medial lobes were fixed in formaldehyde saline (10%) solution for histological analysis; the rest were snap frozen in liquid nitrogen, and stored at -80°C until further use. All animal protocols were approved by the Benaroya Research Institute Animal care and use committee.

**Cell culture of RAW264.7 macrophage cell line:**
RAW 264.7 cells were obtained from ATCC. They were maintained in DMEM, 10% FBS. Cells were grown in complete RAW cell media till 80% confluent. Just prior to the experiment, they were treated overnight in RAW cell media containing 1% FBS. They were then treated for 4 hours with 250 µM ferric ammonium citrate (FAC, Sigma Aldrich, from 50 mM stock of FAC made in sterile water) in the same medium. Treated cells were harvested and assessed for changes in gene expression by quantitative RT-PCR.

**Serum and hepatic biochemical assays:**
Serum and plasma were separated by centrifugation at 4°C and stored at -80°C. Serum glucose, ferritin, liver enzymes namely aspartate aminotransferase and alanine aminotransferase (ALT and AST levels) were determined using the Beckman DXC 800
Analyzer (Brea, CA). Serum hepcidin levels were measured in the serum using the Murine Hepcidin Compete™ ELISA developed by Intrinsic Lifesciences, La Jolla, CA (10). Hepatic malonyldialdehyde (MDA) levels and hepatic triglyceride levels were quantified using the TBARS Assay Kit (Cayman, Ann Arbor, MI) and the Triglyceride Quantification Kit (MBL International, Woburn, MA) respectively, following the manufacturers' instructions. Hepatic non heme iron content (HIC) was measured as previously described (2). Semiquantitative grading of iron was performed on liver sections using Perls' Prussian Blue staining as previously described (26, 14, 20, 2,13).

Histological analysis of liver tissues:
Liver tissues were fixed in formaldehyde saline (10%) solution, and stained with hematoxylin-eosin, Perls' Prussian Blue for iron staining and Masson’s Trichrome to assess fibrosis. Histological scoring was performed by a hepatopathologist (MMY) blinded to treatment assignment for steatosis (0-3), inflammation (0-3), ballooning (0-2), and fibrosis (0-6) along with a diagnosis of not NASH or NASH as previously described (26, 14, 20, 11, 2), with the modification that the steatosis, inflammation and ballooning scores, and iron grades and localization were reported as median scores in Table 1, rather than as averages, in order to maintain them as ordinal variables.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of liver:
The isolation of RNA, the preparation of cDNA, and the RT-PCR reaction was performed as previously described (11). Briefly, total RNA was isolated from liver, adipose tissue and RAW 264.7 macrophage cells using the RNeasy kit (Qiagen,
Valencia, CA). cDNA synthesis was then performed using 1-2 µg of total RNA with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). The PCR reaction mixture contained 125-150 ng cDNA (total volume 5 µL), 1 µL of each of the primers (20 pM), and 10 µL SYBR Green mix (Invitrogen, CA). RT-PCR was performed using an ABI 7900HT instrument (ABI systems, CA). Levels of the target mRNAs were calculated relative to the reference gene, Glyceraldehyde-3 Phosphate dehydrogenase (GAPDH), using the ΔΔCt formula. Several RT-PCR primer sequences have been described previously (11), and the rest are available upon request.

**Western blot analysis:**

Procedures related to protein separation and western blotting have been described (11). Briefly, protein was extracted from liver samples and quantified (Micro BCA Protein Assay Kit; Pierce, Rockford, IL). Equal amounts of protein lysates (~20 µg) were used for immunoblotting using gradient 4-20% Tris-Glycine gels (Biorad, Hercules, CA). Blots were probed using the following primary antibodies: phospho- (thr-172)-AMPKα (Cell Signaling Technologies, Danvers, MA), PGC1α antibodies (1:500, Abcam, Cambridge, MA), MAC-2 (1:1000, Cedarlane), CD68 (Abcam), GAPDH at 1:1000 dilution (Cell Signaling Technologies, Danvers, MA) or phospho( ser-727) -STAT-3 (Cell Signaling Technologies). Corresponding secondary antibodies were obtained from Santa Cruz Biotechnology, CA.

**Statistical Analysis:**

Statistical analysis was performed using GraphPad version 5.0 (Palo Alto, CA). All values below P<0.05 were considered significant. Values are represented as mean
±SEM, unless otherwise mentioned. For the normally distributed populations involving two group comparisons, Student’s t-test were performed; for multiple comparisons, one-way Anova followed by post-hoc tests were performed and for non-normally distributed populations, Mann-Whitney tests were performed.

Results:

Effect of dietary iron on body and liver weight:

Relative to chow-fed animals whose average weight was 59.42±1.19 g, the average weight of the dietary iron (DI)-fed mice was 56.56±1.13 g. Despite the reduced body weights in the DI-fed mice, they were not significantly different (p=0.06). There was no significant change in the liver mass (chow-fed= 4.65 ±0.12, iron-fed= 4.58±0.14).

Dietary iron overload exacerbates hyperglycemia and leads to elevation of serum liver enzymes:

Relative to the chow-fed animals, DI-fed mice had significantly higher serum glucose levels (126.6±13.4 vs 201.1±22.10mg/dL, Figure 1). Furthermore, serum liver enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were higher in the DI group. ALT levels were significantly increased relative to chow-fed controls (Figure 1).

Dietary Iron excess leads to elevated levels of serum and hepatic markers of iron overload:
Serum hepcidin and serum ferritin levels were significantly enhanced in the DI mice relative to controls (Figure 2A-B). Further, hepatic iron content, hepatic hepcidin (Hamp) and transferrin receptor 1 (TfR1) mRNA expression were significantly higher in the DI group compared to chow-fed controls (Figure 2C-D). Staining with Perls’ Prussian blue revealed hepatic iron overload in the DI mice while chow-fed control mice did not demonstrate iron staining (Figure 2E). The distribution of hepatic iron was largely in the RES compartment with a much lower hepatocellular (HC or parenchymal) distribution (Table 1, Figure 2E). In addition, the grade of iron staining in the RES compartment was significantly greater than the HC compartment (Table 1, Figure 2E).

**Dietary iron overload results in NASH histology:**

We next asked if dietary iron excess led to NASH histology. Dietary iron overload caused significant hepatocellular ballooning in 87.5% of the DI-fed mice compared to 20% chow-fed controls (Table 1, Figure 3A-C); while the scores for steatosis and inflammation were not significantly altered between DI and chow-fed mice (Table 1). The mean histology score for ballooning showed a significant elevation in the DI mice relative to chow-fed controls (Figure 3C). As a result of the heightened ballooning injury in 7 out of 8 DI mice, the percentage NASH diagnosis was 75% for the DI mice compared to 20% for the chow-fed controls (Table 1, Figure 3).

**Dietary iron overload causes hepatic oxidative stress and NLRP3 inflammasome activation:**
Since iron is a known contributor of oxidative stress (7, 6, 25), we next examined the effect of iron overload on hepatic oxidative stress. We observed that the levels of malonyldialdehyde (MDA, a derivative of lipid peroxidation) were significantly higher in the DI mice relative to chow-fed mice (Figure 4A). Consistent with these findings, hepatic gene expression levels of antioxidant response genes such as heme oxygenase-1 (Hmox1), Glutathione peroxidase-1 (Gpx1) and Nuclear response factor 2 (Nrf2) were significantly augmented (Figure 4B). In addition, we observed a decrease in the UPR (unfolded protein response)/ER stress related transcription factor / oxidative stress regulator, X-box Binding Protein 1 (Xbp1) mRNA expression (16). Further, since oxidative stress is known to activate the nucleotide binding domain and leucine–rich repeat containing (NLR) protein, NLRP3 inflammasome (32), we examined gene expression of Nlrp3 and the cytokine Il18 and found that they were significantly upregulated in the DI mice.

**Excess iron results in impaired hepatic mitochondrial biogenesis and fatty acid β-oxidation pathway:**

Since oxidative stress is closely linked to mitochondrial dysfunction (7, 6, 25), we next interrogated hepatic gene expression levels of signature fatty acid β-oxidation/ lipolytic transcripts such as carnitine palmitoyltransferase 1A (Cpt1α), Acyl-CoA Oxidase1 (Acox1) and lipoprotein lipase (Lpl), and found them to be significantly reduced in the livers of DI- mice relative to the controls (Figure 5A). Further, the mRNA levels of additional regulatory mitochondrial biogenesis and fatty acid β-oxidation genes such as peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (Pgc1α), Nuclear
respiratory factor 1 (Nrf1), Mitochondrial transcription factor A (Tfam) and cytochrome c oxidase subunit IV (Cox4) were significantly diminished in the DI-fed mice (Figure 5B). Consistent with these findings, western blots performed with protein lysates prepared from the livers of chow and DI mice revealed that phosphorylation status of AMPKα at Threonine -172 and PGC-1α protein levels (master regulators of lipid metabolism) were diminished (data not shown), indicative of reduced mitochondrial function/fatty acid β-oxidation in the livers of the mice with iron excess. Consistent with decreased lipolysis, we found hepatic triglycerides significantly elevated in the DI mice relative to chow-fed controls (Figure 5C).

Iron overload leads to inflammatory activation and altered lipid metabolism in the epididymal white adipose tissue:

We next assessed the effect of iron on the epididymal white adipose tissue (EWAT) with respect to lipid metabolism and proinflammatory activation. Dietary iron caused significant upregulation in mRNA expression of several lipid metabolism genes such as stearoyl COA desaturase (Scd1), Acox1, Lpl and diacylglycerol O-acyltransferase 1, a rate-limiting triglyceride synthetic enzyme (Dgat1) (Figure 6A). Further, dietary iron caused inflammatory activation in the EWAT, as indicated by increased expression levels of chemokine Mcp1, its cognate receptor Ccr2, hypoxia inducible factor, Hif1α and reduced levels of the M2 marker, Arginase-1 (Figure 6B).

Iron excess leads to increase in hepatic inflammatory mediators and immune cell activation:
We next examined whether iron overload had an effect on inflammatory activation in the liver. We found that the livers of DI-fed mice displayed increased gene expression levels of several proinflammatory cytokines such as *Il6*, *Tnfα*, *Tlr4* and *Inos* relative to chow-fed mice, associated with an increased M1 macrophage phenotype (Figure 7A). In contrast, the levels of anti-inflammatory or M2-related macrophage markers such as *Mgl1* and *Tgfβ* were reduced in the livers of DI mice (Figure 7B). Western blots performed with liver lysates from chow and dietary-iron fed mice showed that the protein levels of macrophage markers MAC2 and CD68, were elevated in DI mice compared to chow-fed mice, indicating an increase in the macrophage activation levels in response to dietary iron excess (Figure 7C). In keeping with these observations, the phosphorylated levels (Ser727) of STAT3 protein, a mediator of inflammatory activation, was elevated in the liver lysates from DI mice (data not shown). In addition, we found the gene expression level of several immune cell markers such as CD4, CD8 and Interferon (*Ifn*)-γ were upregulated in the livers of the DI mice. Additionally, mRNA levels of a dendritic cell (DC) marker, CD11c (gene name *Itgax*) were enhanced while the expression levels of CD1d, a NKT cell marker, were downregulated by iron administration (Figure 7D).

**Iron loading of RAW 26.7 cells leads to upregulation of inflammatory mediators:**

We utilized the RAW 264.7 cell line as a surrogate for the macrophages in the liver, and asked if iron administration led to inflammatory/immune cell activation, as was observed in the livers of the DI mice. RAW macrophages were treated with 250 µM FAC as described the Materials and Methods. Relative to untreated controls, we found that the
gene expression levels of several chemokine, cytokine and anti-oxidant mediators such as *Inos, Tnfa, Mcp1*, hepcidin (gene name *Hamp*), *Hmox1* and *Tlr4* were upregulated in response to exogenously administered iron (Figure 8).

**Discussion:**

The goal of this study was to assess the effect of iron overload on NASH development in a murine model of obesity and type 2 diabetes. Our previous studies in human subjects with NAFLD have demonstrated a strong relationship between hepatic iron deposition, especially in RES cells to NASH and advanced fibrosis in NAFLD, but these have been cross-sectional studies (26, 14, 7). The goal of the current study was to determine whether hepatic iron loading in the setting of obesity and steatosis causes NASH and to demonstrate the underlying mechanisms of RES-iron mediated NASH.

We found that dietary iron overload in the setting of obesity and diabetes led to nonalcoholic steatohepatitis by causing hepatocellular ballooning injury in the livers of DI mice. Ballooning is a hallmark of human NASH (3, 34), and has been difficult to recapitulate in most experimental animal models of NASH (11). Hepatocellular ballooning is accompanied by significantly increased hepatic oxidative stress and inflammasome activation in the DI mice. There are additional crucial pathways on which dietary iron exerted an effect in this model. The DI mice displayed increased hepatic inflammatory immune cell activation and impaired hepatic mitochondrial fatty acid β-oxidation.

We observed that while the NASH histology scores for steatosis and inflammation were largely unaltered between control and DI-fed mice, hepatic ballooning injury increased
significantly in the DI mice. While it is not clear what cellular mechanisms contribute to hepatocellular ballooning; oxidative stress, impaired proteasomal function and cytoskeletal degradation have been thought to be involved (3, 34). Iron-induced oxidative stress and inflammasome activation could be contributing to the ballooning injury in our model.

DI mice experienced oxidative stress as evidenced by increased levels of MDA and elevated hepatic gene expression levels of known antioxidant stress response genes such \( \text{Hmox1}, \text{Gpx1} \) and \( \text{Nrf2} \). This appears to be a compensatory response to the ROS generated by iron. NRF2 activators have been demonstrated to prevent iron or diet-induced NASH (22, 30). Additionally, Hemeoxygenase-1 has also been shown to play a protective role in concert with adiponectin against iron-induced injury in hepatocytes (15). Furthermore, the mRNA levels of XBP1, the unfolded protein response regulator (UPR) were downregulated in the DI mice. XBP1, in addition to its role in UPR/ER stress, has recently been found to be an oxidative stress regulator (16). Furthermore, NLRP3, an inflammasome danger sensor, thought to be involved in NASH pathogenesis, is believed to be linked closely to oxidative stress (32). Thus, the upregulation of antioxidant genes in our model is indicative of RES iron-mediated oxidative stress related liver injury. This might have contributed the ballooning degeneration that was evident in the DI mice. We observed that 7/8 DI mice had ballooning injury, relative to 1/5 in chow-fed controls. Furthermore, the reduced levels of mitochondrial biogenesis and fatty acid β-oxidation genes such as \( \text{Pgc1}\alpha \) and \( \text{Nrf1} \), and reduced hepatic levels of activated AMPKα and PGC1α proteins in our model suggests that the mice experienced impaired mitochondrial function. Oxidative stress is known to
lead to mitochondrial dysfunction. Iron-induced oxidative stress, inflammasome
activation and mitochondrial dysfunction could be the primary injury in this mouse
model, contributing to NASH.

Iron overload had additional crucial effects on the iron-fed mice, in that DI mice
showed immune cell activation in the liver, as evidenced by the activated status of
macrophages, T cells and dendritic cell (DC) marker(s), but reduced expression of a
known beneficial natural killer T (NKT) cell marker, CD1d. While the exact role of NKT
cells in NASH is not yet completely elucidated, CD1d null mice have been reported to
be predisposed to diet-induced metabolic disorders (21). Interestingly, while we did not
see an alteration in the inflammation score in the NASH histology, there were significant
changes in the gene expression levels and the protein levels of key immune cell
markers, which have been shown to be the important for NASH progression,
demonstrating that the immune cells get activated or altered by iron overload in our
model. These observations suggest gene expression analysis might be a more sensitive
readout of immune cell activation, rather that histological analysis. Consistent with these
in vivo findings, RAW macrophages treated with iron showed increased proinflammatory
gene expression changes.

Iron was restricted to predominantly RES localization in the livers of DI mice. We
have previously demonstrated in several cross-sectional human studies that RES
localization of iron is associated with an increased severity of NASH and advanced
fibrosis (26), and increased oxidative stress and apoptosis (20); important features of
NASH (26, 14, 20). Furthermore, in vitro studies with iron-loaded Kupffer cells have
previously shown that iron causes the activation of NF-κB, a potent inflammatory
mediator, implicated in the pathogenesis of NASH (4). Thus, in our model, the predominant localization of iron in the macrophages; and the increased inflammatory activation of RAW macrophages upon iron-loading points to the role of iron in mediating its effect on hepatic macrophages by causing and augmenting hepatic inflammation.

Kupffer cells and recruited hepatic macrophages (RHM) have been shown to be critical for the pathogenesis of NASH in several key rodent studies (31, 23). An increase in the polarization of macrophages towards the more “proinflammatory M1- or classically activated” phenotype and the reduction in the anti-inflammatory M2 phenotype has been causally linked to adipose tissue inflammation and obesity (18). Furthermore, studies are emerging that indicate that a similar phenotype switch promoting proinflammatory M1 macrophage polarization and limiting M2 macrophage populations occurs in the liver as it progresses from benign fatty liver to nonalcoholic steatohepatitis (31, 23, 9). In our model, iron appears to be the trigger to cause the switch in polarization towards the M1 macrophages. In addition, CD8 T cells, which are one of the main producers of the potent cytokine, interferon γ, were upregulated in the DI mice, and been implicated in the mechanisms leading to NASH (19, 28). Additionally, dendritic cells, a type of antigen presenting myeloid immune cell population (characterized by the presence of CD11c marker, which was elevated in the livers of the DI mice), have also been proposed to contribute to the pathogenic process that governs NASH (12). The livers of DI mice may have experienced hepatocellular injury and necroinflammation, not only as a result of hepatic oxidative damage, inflammasome activation and accompanying mitochondrial dysfunction, but also due to the activation and alterations in key hepatic immune cell
populations, and the associated production of signature proinflammatory M1 cytokines and chemokines and diminishing M2 markers in our model.

In addition to the above findings, we observed that the iron-supplemented mice displayed increased metabolic impairments. While the genetically obese and diabetic Lepr\(^{db/db}\) mice are hyperglycemic at baseline; the mice supplemented with iron showed a significant increase in their fasting glucose levels, elevated liver function tests, increased oxidative stress levels as evidenced by increased MDA and elevated serum hepcidin and ferritin levels. These findings are consistent with previous iron overload studies, which have demonstrated systemic effects of iron on several metabolic parameters (7, 6, 25, 5). These data suggest that dietary iron exacerbates systemic inflammation and metabolic impairments in this genetic model of ‘diabesity’, contributing to the worsening of the metabolic syndrome and the NAFLD in these mice. Increased serum ferritin has been associated with advanced NAFLD with severe fibrosis in our studies on patients (14). Furthermore, we have also demonstrated that increased serum hepcidin has been associated with increased RES iron in NAFLD patients (24). We believe that the increased levels of iron deposition in the RES compartment could be a consequence of the elevated serum hepcidin levels in response to iron-induced inflammation in our model. This predominantly hepatic RES deposition in response to 2% carbonyl iron is consistent with a previous report (5). Increased serum hepcidin has also been associated with metabolic syndrome in humans (29).

We also observed that the hepatic and adipose lipid metabolism was impaired in our DI mice. Increased expression of lipolytic and triglyceride synthetic genes in the visceral adipose tissue in our model may have contributed to enhanced levels of free
fatty acids. Consistent with these observations, we found that the hepatic triglycerides levels were elevated in the dietary iron-supplemented mice.

While iron overload has been demonstrated in a rodent model to cause oxidative stress and fibrogenesis (1), and obesity has been shown to alter iron homeostasis in the adipose tissue macrophages (27); and the interplay between iron overload/homeostasis, metabolic syndrome and NASH has been reviewed previously (33, 8); to our knowledge, this is the first study examining the effect of iron overload on hepatic immune cells activation and its relation to NASH. In this study, we show that iron overload leads to alterations in liver immune cell populations such as recruited and resident macrophages, lymphocytes (CD4 and CD8), dendritic cells and NK cells. Our study is unique also in demonstrating that iron in and of itself causes the hepatic and adipose tissue macrophages to be M1 polarized.

In summary, we have demonstrated that dietary iron in the context of genetic obesity, with the attendant constellation of metabolic dysfunction, can accelerate the progression of NAFL to NASH due to adipose tissue dysfunction, RES deposition of hepatic iron, hepatic oxidative stress, inflammation associated with immune cell activation and ballooning injury as described in the proposed model. Studies aimed at better understanding molecular mechanisms underlying and mitigating iron overload will be important next steps towards developing effective therapeutic interventions for iron-mediated NASH.
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Table and Figure legends:

**Table 1: NASH histology and iron localization for chow and DI-fed mice:** Steatosis, ballooning and inflammation, and iron grade and localization in the reticuloendothelial system (RES) and the hepatocellular (HC) compartment are reported as median scores for chow and DI mice, following the staging and grading criteria of the NASH CRN. N=5 chow-fed, N=8, dietary iron fed mice.

**Figure 1: Dietary iron causes metabolic dysfunction:** Chow (Normal chow, NC) and dietary iron-fed mice (DI) were examined with respect to glucose, serum alanine amino transferase (ALT) and serum aspartate amino transferase (AST) levels. N=5 chow-fed, N=8, dietary iron fed mice. * P<0.05

**Figure 2: Effect of dietary iron overload on serum and hepatic markers of iron homeostasis:** A. Representative Perls’ Prussian Blue staining for Iron on chow and DI–fed mice at a magnification of 630X. N=5 chow-fed, N=8, dietary iron fed mice. B. Serum hepcidin was determined by performing serum ELISA. C. Serum ferritin was determined using the Beckman Bioanalyzer. D. Hepatic iron concentration in the chow and iron-fed mice was determined using a biochemical assay. E. Gene expression levels of hepatic hepcidin and Transferrin Receptor 1 in the livers of chow and dietary iron fed mice. * P<0.05, **P<0.01, ***P<0.001

**Figure 3: Effect of dietary iron overload on hepatocellular ballooning:** A. H&E stained liver histological images of representative chow (left) and DI–fed mice(right) at 200X magnification. The arrows point towards ballooning injury in the liver of the DI-fed mouse. B. Representative H&E staining for chow and DI–fed mice, at a magnification of
Figure 4: Dietary iron leads to oxidative stress and inflammasome activation in the liver.

A. Lysates from the livers from chow and dietary iron-fed mice were subjected to a TBARS assay to determine the presence of reactive oxygen species evidenced by the production of malondialdehyde (MDA). B. mRNA expression levels of oxidative stress genes such as GPx1, Hmox1, Nrf2, Xbp1, and inflammasome markers such as Nlrp3 and Il18 were determined by RT-PCR in the chow and dietary iron–fed mice. N=5 chow-fed, N=5-8, dietary iron fed mice. * P<0.05, **P<0.01

Figure 5: Effect of dietary iron overload on hepatic fatty acid β-oxidation and mitochondrial biogenesis pathways.

A. mRNA levels of hepatic fatty acid β-oxidation and lipolytic genes were assessed in the livers of the chow and dietary iron-fed mice. B. mRNA levels of hepatic mitochondrial biogenesis-related genes such as Nrf1, Tfam1, Pgc1α and Cox4 were assessed in the livers of the chow and dietary iron-fed mice. C. Hepatic triglyceride levels in the livers of chow and DI mice. N=5 chow-fed, N=5-8, dietary iron fed mice. * P<0.05.

Figure 6: Effect of iron on lipid metabolism and inflammatory activation in epididymal white adipose tissue (EWAT).

A. Gene expression levels of lipid metabolism genes-Scd1, Acox1, Lpl and Dgat1. B. Gene expression levels of inflammatory markers in chow and dietary iron-fed mice from EWAT - Mcp1, Ccr2, Arginase1 and Hif1α. N=5 chow-fed, N=8, dietary iron fed mice. * P<0.05
Figure 7: Effect of iron excess on hepatic inflammation and immune cell activation: A. Gene expression levels of M1 markers such as Inos, Tlr4, Il6, Tnfa and M2 markers such as Mgl1 and Tgfβ in chow and DI-fed mice were examined. B. Liver lysates were assessed for the expression of CD68 and MAC-2, two macrophage specific markers. C. mRNA levels of immune markers such as CD11c (Itgax), CD1d, CD4, CD8, Ifnγ. N=5 chow-fed, N=5-8, dietary iron fed mice. * P<0.05

Figure 8: Effect of iron excess on RAW 264.7 macrophages: RNA from untreated and iron-treated RAW cells was assessed for gene expression changes related to signatures of inflammation and oxidative stress such as Inos, Tnfa, Mcp1, hepcidin (Hamp), Hmox1 and Tlr4. N=3-5. * P<0.05, **P<0.01

Figure 9: Proposed model for the mechanisms by which iron could influence the adipose tissue and the liver in the genetically obese diabetic mice to cause nonalcoholic steatohepatitis: Iron overload leads to impaired lipid metabolism and inflammatory activation in the visceral adipose tissue. In the liver, dietary iron causes alteration in lipid metabolism, oxidative stress, M1 inflammatory activation, alteration in hepatic immune cells leading to hepatocellular ballooning injury and thereby NASH.
Figure 2

A. Serum Ferritin

B. Serum Hepcidin

C. Serum Ferritin

D. Hepatic Iron

E. HAMP

Transferrin Receptor
Lipid metabolism genes

A. Relative mRNA expression for CPT1α, LPL, and ACOX.

B. Mitochondrial biogenesis related genes

C. Hepatic triglycerides

Figure 4
Figure 5
A. Lipid metabolism related

B. M1 activation and inflammation related

Figure 6
M1 markers

**A.**

- **IL-6**
- **TNFα**
- **INOS**
- **TLR4**

M2 macrophage markers

**B.**

- **TGFβ**
- **MGL1**

C. Immune cell markers

- **CD1d**
- **CD11C**
- **IFNγ**
- **CD8**
- **CD4**

**Figure 7**
Figure 8
Proposed schema for iron-related multiple mechanisms leading to (ballooning injury &) NASH in obese diabetic mice

IRON

OXIDATIVE STRESS

M1 & IMMUNE CELL ACTIVATION

IMPAIRED LIPID METABOLISM

ADIPOSE TISSUE

PROINFLAMMATORY ACTIVATION

ALTERED LIPID METABOLISM

INVOLVEMENT

LIVER

BALLOONING

INFLAMMATION

Figure 9
Table 1: NASH histology and hepatic iron grade and distribution:

NASH CRN grading criteria were used to determine NASH histology and the iron grade and distribution in the hepatocellular (HC) and reticuloendothelial system (RES) compartments, in the livers of the chow and dietary iron-fed mice and reported as median scores. N=5 chow-fed mice and N=8 dietary iron-fed mice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chow</th>
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<tr>
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<tr>
<td>Hepatic RES Iron grade</td>
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</tr>
<tr>
<td>Hepatic HC Iron distribution</td>
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<tr>
<td>Hepatic RES Iron distribution</td>
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