Hepatic iron is the major determinant of serum ferritin in NAFLD patients

Running title: Ferritin reflects liver iron in NAFLD

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Abbreviations

CRP, C-reactive protein; HIC, hepatic iron concentration; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; GDF-15, growth differentiation factor-15; MRS, magnetic resonance spectroscopy; HLC, hepatic lipid content; DIOS, dysmetabolic iron overload syndrome.

Conflict of Interest

MP, RB, SN and EB are shareholders in Perspectum Diagnostics, a University of Oxford spin-out company. RB and SN are on the board of directors. RB is an employee of Perspectum Diagnostics. MP, RB, SN and EB have patent applications for use of MRI for the assessment of liver disease. All other authors have no conflict of interest.

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Abstract

Background and Aims

Elevated serum ferritin is common in NAFLD, and is associated with more advanced disease and increased mortality. Hyperferritinemia in NAFLD is often attributed to inflammation, while in other conditions ferritin closely reflects body iron stores. The aim of this study was to clarify the underlying cause of hyperferritinemia in NAFLD.

Methods

Ferritin levels were examined with markers of iron status, inflammation and liver injury across the clinical spectrum of NAFLD using blood, tissue and magnetic resonance (MR) imaging. A separate larger group of NAFLD patients with hepatic iron staining and quantification were used for validation.

Results

Serum ferritin correlated closely with the iron regulatory hormone hepcidin, and liver iron levels determined by MR. Furthermore, ferritin levels reflected lower serum adiponectin, a marker of insulin resistance, and liver fat, but not cytokine or CRP levels. Ferritin levels differed according to fibrosis stage, increasing from early to moderate disease, and declining in cirrhosis. A similar pattern was found in the validation cohort of NAFLD patients, where ferritin levels were highest in those with macrophage iron deposition. Multivariate analysis revealed liver iron and hepcidin levels as the major determinants of serum ferritin.

Conclusions

While hyperferritinaemia is associated with markers of liver injury and insulin resistance, serum hepcidin and hepatic iron are the strongest predictors of ferritin

levels. These findings highlight the role of disordered iron homeostasis in the pathogenesis of NAFLD, suggesting that therapies aimed at correcting iron metabolism may be beneficial.

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Key words

Ferritin, hepcidin, MRI, NAFLD, liver iron

Key points

- Elevated serum ferritin is found in approximately 30% of NAFLD patients, and is associated with more advanced disease and increased mortality.
- This study demonstrates that liver iron, and the location of iron deposition, is the chief influence on ferritin levels in NAFLD, with macrophage iron associated with higher ferritin levels.
- Serum ferritin and hepcidin change significantly across fibrosis stages in NAFLD, increasing in intermediate stages and declining in cirrhosis.
- We demonstrate an association iron status and adiponectin, a marker of insulin resistance, including a novel association between liver iron and adiponectin levels.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the commonest liver disease in Western clinical practice, representing the hepatic manifestation of the obesity pandemic. With increasing weight and adiposity, the liver becomes congested with fat, resulting in

inflammation and hepatocellular damage (termed non-alcoholic steatohepatitis, NASH), potentially progressing to fibrosis, cirrhosis and primary liver cancer. The identification of patients at risk of progression is vital to disease management. While the presence of hepatic fibrosis on liver biopsy is the single most important predictor of outcome in NAFLD,(1, 2) non-invasive surrogates of fibrosis are increasingly employed to avoid the risks and costs associated with liver biopsy.(3)

NAFLD is an asymptomatic condition of insidious onset, often identified incidentally by blood testing or imaging. About half of male patients have raised serum ferritin levels.(4) Indeed, mild hyperferritinaemia is often the only presenting abnormality leading to a diagnosis of NAFLD.(5) Elevated ferritin has been identified as a predictor of advanced fibrosis in several large cohort studies,(6-8) and more recently predicted mortality in 222 NAFLD patients followed up for 15 years.(9) Furthermore, hyperferritinaemia has been associated with obesity, insulin resistance, and cardiovascular disease, conditions inherently related to NAFLD.(10)

Although ferritin is the chief iron storage protein and can closely reflect body iron stores, it is an acute phase reactant, leading to difficulty in its interpretation in the presence of co-existing liver injury. In NAFLD, mild hepatic iron deposition is seen in up to 30% of patients,(11) while increased hepatic and systemic inflammation are also common. Iron has been implicated in cellular oxidative stress and insulin resistance, key features of NAFLD pathogenesis, and hepatic iron deposition has been associated with advanced fibrosis.(12, 13)

Despite its widespread availability, ferritin is not routinely used as a diagnostic aid in NAFLD, in part due to uncertainty as to whether ferritin reflects liver damage, inflammation or iron deposition. In this study, a multi-faceted approach combining blood, histological, and imaging markers of iron status, inflammation, and disease stage was used to determine the influences on ferritin levels in NAFLD patients. Using independent discovery and validation cohorts, we demonstrate that hepatic iron, rather than other markers of liver damage, is the predominant determinant of serum ferritin levels.

Patients and Methods

Patient characteristics

MRI Cohort

Study participants were prospectively recruited between June 2011 and October 2014, as part of a study using multiparametric magnetic resonance (MR) imaging in liver disease, described elsewhere.(14) 51 NAFLD patients were eligible for inclusion. Baseline characteristics were compared with 30 patients with viral hepatitis (20 Hepatitis C, HCV, and 10 hepatitis B, HBV) and 20 healthy adult controls (Table 1). All individuals underwent physical examination, anthropometry, blood sampling and transient elastography (Fibroscan®). NAFLD and viral hepatitis patients underwent a liver biopsy and multiparametric MRI, while healthy controls had an MRI. All study participants gave written informed consent. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the institutional research departments and the National Research Ethics Service (11/H0504/2, 13/SC/0243; registered clinical trial (NCT01543646)).

Definitions and criteria

Diagnosis of NAFLD was confirmed by steatosis affecting ≥5% of hepatocytes on liver biopsy, in the absence of confounding factors such as alcohol excess or other causes of chronic liver injury. Patients with excess alcohol consumption (>20g alcohol/day), iron storage disorders (including hereditary or transfusional iron overload), or recent gastrointestinal bleeding, were excluded.

Laboratory Tests

Blood samples were obtained after an overnight fast. Routine haematological and biochemical tests were performed in the clinical laboratories of the John Radcliffe Hospital, Oxford. Patients with hyperferritinaemia (>300μg/L for men, 200μg/L for women) and transferrin saturation >45% underwent screening for HFE mutations.

Histological assessment of liver biopsy samples

The median biopsy length was 17mm (IQR 14-24), with a median of 10 (IQR 8-15) portal tracts. Liver biopsies were assessed by an independent histopathologist, blinded to MR data (LMW). NAFLD Fibrosis stage was determined as outlined by Brunt (stages 0-4; F0, no fibrosis; F1, perisinusoidal/portal fibrosis; F2, perisinusoidal and portal/periportal fibrosis; F3, septal or bridging fibrosis; and F4, cirrhosis).(15) Viral hepatitis samples were assessed for fibrosis by the Ishak stage (F0–F6). Hepatic lipid content reflected the percentage of hepatocytes with lipid vesicles, graded as 0 (<5%), 1 (5-<33%), 2 (33-<66%) and 3 (>66%). Hepatic iron deposition was estimated using Perls' stain and semi-quantified using a five tier grading system (0: no hemosiderosis to 4: severe hemosiderosis) as described by Scheuer,(16) and the location of iron deposition was either hepatocellular, reticulo-endothelial (Kupffer) or mixed.

Hepcidin, cytokine and adipokine measurement

Serum hepcidin was assayed using the Human Hepcidin-25 EIA Kit (Bachem®) according to the manufacturer's protocol, as described elsewhere.(17) Serum total adiponectin (APN) was measured using the Quantikine® ELISA□Human Total Adiponectin/Acrp30 Immunoassay (R&D Systems) according to the manufacturer's protocol. Serum levels of insulin, leptin, glucagon, IL1β, IL6, IL8, TNFα, IL10, IL22, IFNγ, PC9, IP-10, MCP-1, E-selectin, ICAM1, Osteopontin, HGF, Chi3L1, TIMP-1 and GDF-15 were simultaneously quantified using an electrochemiluminescence assay (Luminex®, R&D Systems). Aminoterminal peptide of pro-collagen III (PIIINP) levels were measured using an ADVIA Centaur XP automated immunoanalyzer (Siemens).

MR imaging parameters

Participants underwent non-contrast multiparametric MR to detect liver fibrosis, steatosis and hemosiderosis, described elsewhere.(14) Briefly, scans were performed on a 3-Tesla scanner (Tim Trio, Siemens). Transverse abdominal T_2□ MR maps through the liver and spleen were acquired for estimating iron content. T_2□ relaxation time (ms) is inversely related to iron content, meaning higher liver iron levels yield lower T2* values. Proton magnetic resonance spectroscopy (^{1}H-MRS) was used to measure liver fat content, expressed as a percentage (%) of the total water signal. Scans were performed after a ≥4 hour fast.

Transient Elastography

Transient elastography (Fibroscan®, Echosens) was performed following an overnight fast. Median liver stiffness measurements (LSM) were expressed in kiloPascals (kPa). Procedures with ≥10 successful acquisitions, success rate >60% and inter quartile range <30% of the median were included.

Validation cohort

A validation cohort of 404 patients with histologically confirmed NAFLD were consecutively evaluated between January 2005 and December 2013 at a referral center for iron overload and metabolic liver diseases in Northern Italy. Part of this group was described previously.(18) Hepatic iron staining was performed according to Scheuer,(16) in patients from a previous study or when hyperferritinemia or NASH were present.(13) Hepatic iron quantification by atomic absorption spectrometry was performed in 129 patients. C282Y and H63D HFE mutations were determined in 380 cases, including all patients with hyperferritinemia and/or positive iron staining. HFE genotypes potentially predisposing to iron overload (C282Y/H63D, H63D+/+ or C282Y/wt) were detected in 20 cases (5%). C282Y homozygotes were excluded from the study. The study protocol was approved by the Institutional Review Board of the Fondazione IRCCS Ca' Granda, and conformed to the Declaration of Helsinki. Written informed consent was obtained from each patient.

Statistical analysis

Continuous, normally distributed variables were reported as mean +/-standard deviation (SD), or median (range) for non-Gaussian distribution, and (base 10) log-transformed before analysis. Comparison between groups was performed using analysis of variance (ANOVA) or Kruskal-Wallis test for continuous variables, or

Chi-square or Fisher's exact test for categorical variables. Correlations were performed by Pearson's or Spearman Rank method, where appropriate. A receiver operating characteristic (ROC) analysis was performed to determine the diagnostic ability of ferritin for the detection of hepatic siderosis. Multiple linear regression was performed to assess for variables associated with ferritin, and direct logistic regression to determine predictors of NAFLD fibrosis stage, inputting variables associated with the dependent variable at univariate analysis with a significance cutoff of $p<0.1$. Data analysis was performed using SPSS Statistics 23 and Graphpad Prism 6.0. A p-value of <0.05 was considered statistically significant.

Results

Subject characteristics

Table 1 depicts the baseline characteristics of study subjects from the MRI cohort, comparing NAFLD patients with viral hepatitis and controls. As expected, patients with NAFLD had higher BMIs, along with higher serum insulin, leptin, HOMA-IR values and liver fat determined by MR spectroscopy, and lower serum total adiponectin levels, consistent with insulin resistance (all $p<0.0001$). No significant difference in overall disease stage at biopsy or liver stiffness measurement was noted between NAFLD and viral hepatitis groups.

Serum ferritin and iron status

Table 2 shows differences in serum, histological and imaging markers of iron status between groups. Median serum ferritin was higher in NAFLD and viral hepatitis patients compared to controls ($p=0.0045$, Fig.1A), while serum iron and transferrin saturation were significantly lower in NAFLD than other groups. Serum hepcidin

levels did not differ for NAFLD compared to viral hepatitis patients (non-significant trend for elevated hepcidin in NAFLD; p=0.06). Compared with viral hepatitis and controls, hepatic iron levels were significantly elevated in NAFLD, indicated by lower T2* values (p=0.0025, Fig.1B), although differences in gender distribution between groups must be considered when interpreting these data. Ferritin and hepcidin correlated closely in NAFLD patients (r=0.73, p<0.0001, Fig.1C). Furthermore, a significant inverse correlation between ferritin and liver T2* was noted (r=-0.76, p<0.0001, Fig.1D), and hepcidin and liver T2* (r=-0.6, p<0.0001, Fig.1E), indicating that these markers closely reflect liver iron levels in NAFLD. Ferritin also correlated with hemoglobin (r=0.46, p=0.001), suggesting that levels reflect increased iron availability for erythropoiesis. In viral hepatitis patients, ferritin did not correlate significantly with hepcidin (r=-0.33, p= 0.67) or liver T2* (r=-0.33, p=0.07), while in controls, ferritin correlated with hepcidin (r=0.63, p=0.003), and liver T2* (r=-0.498, p=0.026). Seven NAFLD patients had hyperferritinaemia and transferrin saturation >45%; Four screened negative for HFE mutations, and one C282Y/H63D heterozygote and 2 C282Y heterozygotes were identified. Exclusion of the compound heterozygote did not significantly alter the results outlined.

Clinical features of the independent validation cohort are outlined in Table S1. Within this cohort, iron staining was more frequently detected in non-parenchymal than parenchymal cells (n=164, 41% vs. n=120, 30%, p<0.0001). However, in patients with HIC measurement available, ferritin increased with the severity of both parenchymal and non-parenchymal iron deposition (p<0.0001, Fig S1A and S1B), reflecting increased HIC in both cases (p<0.0001 for all, Fig S1C and S1D), with significantly higher ferritin levels in non-parenchymal than parenchymal siderosis,

despite no difference in hepatic iron concentration between groups. However, in most cases both parenchymal and non-parenchymal siderosis coexisted (n=103, 25% mixed vs. 61 (15%) non-parenchymal alone, vs. 17 (4%) parenchymal alone, p<0.0001; 223 patients (56%), had no siderosis). A ferritin cut-off of 378 μg/L for detecting siderosis yielded a sensitivity of 76% and specificity of 75%, and a ROC value of 0.78 (indicating moderate accuracy). In the subgroup of 129 NAFLD patients with HIC measurement, ferritin correlated significantly with hepatic iron concentration (r=0.28, p=0.004, Fig 1F).

Serum ferritin, insulin resistance and hepatic steatosis

Serum ferritin levels increase alongside components of the metabolic syndrome,(10) which accumulate with increasing NAFLD severity. While no significant association between ferritin and anthropometric measurements was seen in NAFLD (including weight, BMI, hip and waist circumference), a strong positive correlation between ferritin and liver steatosis as measured by MR (HLC) or histologically, was found (r=0.57, p<0.0001, Fig.2A and r=0.5, p=0.0002, respectively), with similar trends for hepcidin (r=0.42, p=0.0024; Fig.2B and r=0.4, p=0.0034). Ferritin and hepcidin correlated with total adiponectin levels (r=-0.27, p=0.05 and r=-0.28, p=0.049, respectively; Fig.2C and 2D) and with fasting glucose (r=0.29, p=0.041 and r=0.4, p=0.003, respectively) while increasing liver iron (by MR) correlated significantly with reduced adiponectin levels (r=0.39, p=0.0049; Fig.2E). No association between ferritin and insulin, HOMA-IR or leptin levels were evident. In viral hepatitis patients and healthy controls, ferritin did not correlate significantly with these metabolic features. In the validation cohort, no significant correlation was detected between ferritin and histological grade of steatosis (R=0.05; p=0.33).

Serum ferritin and markers of inflammation

Hyperferritinemia in NAFLD is often attributed to systemic or hepatic inflammation. In the MRI cohort, no association was noted between ferritin and CRP levels (r=-0.22, p=0.12), or inflammatory grade on liver biopsy (Rho=0.07, p=0.62), or serum levels of IL-6, PC9, MCP-1, E-selectin, Osteopontin, ICAM-1, IP-10 (Figure S2A-E). A significant negative correlation between GDF15, a stress-responsive molecule implicated in iron homeostasis and hypoxia,(19) and both ferritin and hepcidin was noted (r=-0.36, p=0.009, Figure S2F and r=-0.6, p<0.00001, respectively). Levels of IL-1β, TNFα, IL-10, IL-22, and IFNγ were not reliably detectable in serum from NAFLD patients. In the validation cohort, ferritin correlated with lobular necroinflammation (R=0.11; p=0.021), but not with hepatocellular ballooning (R=0.02; p=0.71).

Serum ferritin and fibrosis stage

Several studies have reported an association between serum ferritin and advanced liver disease in NAFLD.(6-8) As depicted in Figure 3, ferritin differed according to fibrosis stage, whereby ferritin increased significantly from F0/1 to F2, then decreased through latter stages (F3 and F4; Fig. 3A). The distribution of hyperferritinaemia across fibrosis stages were: F0/1, 4/17 (23.5%); F2, 9/16 (56.3%); F3, 3/9 (33.3%); and F4, 0/9 (0%). Multiple logistic regression revealed ferritin and hepcidin to be independent predictors of early (F0/1) versus significant (F2) fibrosis stage (Table S2). This pattern was mirrored by serum hepcidin, liver iron and steatosis, which increased in early fibrosis stages and fell in advanced disease (Figure 3B-D). A stepwise and significant increase in splenic T2*, suggestive of decreasing iron, was

noted as disease progressed (Fig. 3E). Ferritin levels did not correlate significantly with blood-based fibrosis markers (TIMP1, P3NP, or Chi3-L1). Separating patients with and without NASH did not reveal differences in iron markers, except for hepcidin which was significantly higher in NASH patients (Table S3). In the validation cohort, a similar pattern of ferritin levels was noted, whereby ferritin increased significantly from F0/1 stage to F3 ($p=0.013$), and then decreased ($p=0.048$) in cirrhosis (Fig.3F). Exclusion of patients with HFE mutations did not alter these results significantly (Fig S3).

Determinants of serum ferritin using regression analysis

In order to determine which factors influence ferritin levels in NAFLD, univariate and multivariate regression analyses were performed. Notably, age and gender were not significantly associated with ferritin in the MRI cohort. In fact, only liver iron as determined by MR (T2*) and hepcidin were independent predictors of ferritin levels (Table 3). A model combining liver T2*, hepatic lipid content (HLC), hemoglobin (Hb), alanine aminotransferase (ALT) and hepcidin accounted for 67% of the variance in ferritin levels. There was no evidence of multicollinearity between variables. In the validation cohort, multivariate regression revealed that ferritin was independently associated with mostly non-parenchymal, but also parenchymal iron deposition, older age, male sex, and hepatic necroinflammation (Table S4). Removing patients with HFE mutations did not alter these results significantly.

Discussion

This study further clarifies the basis for hyperferritinemia in NAFLD, by examining the potential determinants of serum ferritin in patients using a multi-faceted approach.

The results highlight the complex interaction between co-factors propagating liver injury and disease progression in NAFLD. Most of the study subjects did not have significant hyperferritinemia or iron overload. Instead, subtle changes in iron status were evident, which correlated with disease stage.

Competing factors may influence ferritin levels in NAFLD and viral hepatitis, as no difference in median serum ferritin was seen despite differing liver iron levels. Close correlations between ferritin, hepcidin and liver iron were evident in NAFLD, but not in viral hepatitis. Multivariate analysis revealed hepcidin and liver iron as the major determinants of ferritin in NAFLD. Importantly, the association between ferritin and liver iron was confirmed in a larger, independent cohort of NAFLD patients. Consistent with previous reports, iron deposition in NAFLD had a predominantly non-parenchymal or mixed non-parenchymal and hepatocellular distribution.(13, 20) Ferritin levels more closely reflected non-parenchymal than hepatocellular iron deposition. Whether or not this reflects iron phagocytosed from necrotic hepatocytes, erythrophagocytosis, or iron actively redistributed into macrophages by hepcidin, and active secretion of ferritin from macrophages,(21) remains to be determined. Furthermore, Kupffer cell iron has been associated with increased oxidative stress and apoptosis in NAFLD,(22) highlighting the clinical relevance of hyperferritinemia in NAFLD.

The close relationship between ferritin and liver iron challenges the dogma that hyperferritinemia in NAFLD and the metabolic syndrome relates to inflammation alone.(6) Using a different approach, Beaton and colleagues assessed changes in hepatic iron concentration in paired liver biopsies before and after quantitative

phlebotomy in 56 NAFLD patients, and similarly found a close correlation between ferritin and hepatic iron removed, rather than CRP or ESR.(23) While ferritin levels reflect liver iron in NAFLD, the ratio of ferritin to liver iron appears to be higher in NAFLD than in hereditary haemochromatosis,(24) possibly reflecting differences in the pattern of iron deposition and the co-existence of hepatic steatosis.(25)

Although increased liver iron in hereditary hemochromatosis results from reduced hepcidin causing excessive dietary iron absorption, an appropriate increase in hepcidin in response to increased liver iron was seen in NAFLD, supporting findings reported elsewhere.(26) Furthermore, serum iron and transferrin levels were lower in NAFLD patients compared to viral hepatitis and controls, and this along with a predominance of reticulo-endothelial iron draws comparisons with the anaemia of chronic disease where iron is sequestered in macrophages. This is also evident in obesity, which is corrected following weight loss surgery.(27) This effect should limit systemic iron levels. Instead, a failure of hepcidin action ('hepcidin resistance') may prevail,(28) leading to increased liver iron, with augmented iron absorption reported in NASH patients, despite elevated hepcidin levels.(29)

In the discovery cohort, hepatic iron levels were determined by MR, an excellent indicator of liver iron concentration, even in the presence of steatosis.(30) Given the sampling variability associated with liver biopsy and the patchiness of hepatic iron deposition,(31) MR provides an advantage over liver biopsy in the assessment of liver iron. In contrast, hepatic staining in the validation cohort allowed qualitative assessment of iron distribution, which was not possible by MR.

The NAFLD fibrosis subgroups were evenly balanced, allowing meaningful comparisons. A clear variation in ferritin became evident upon the separation of NAFLD by fibrosis severity, with increased levels from mild to moderate disease, declining in advanced fibrosis. The subsequent reduction in ferritin in cirrhosis explains its limitations as a non-invasive predictor of fibrosis.(32) Lower ferritin and hepcidin noted in cirrhosis may relate to increased bone marrow iron demand from portal hypertension and covert gastro-intestinal blood loss. Elevated GDF15, a marker of erythroid drive, correlated closely with iron markers in advanced disease, and with hemoglobin, supporting this hypothesis. Another explanation for ferritin/hepcidin decline in advanced fibrosis could reflect reduced liver fat and inflammation in 'burnt-out' NASH. Indeed, lobular necroinflammation contributed to ferritin levels in the validation cohort. In early NAFLD, increased hepatic iron may promote liver injury and fibrogenesis, as ferritin and hepcidin independently predicted the presence of significant fibrosis. This suggests that therapeutic strategies modulating iron homeostasis should target early/intermediate disease stages.

Several studies have suggested a role for iron in insulin resistance, a key pathogenic feature of NAFLD.(10) Much like hyperinsulinemia promotes hepatic lipogenesis, elevated insulin may induce hepatic iron deposition by increasing hepatocyte iron uptake.(33) Furthermore, ferritin and hepcidin correlated with hepatic lipid content on MRI, and with reduced adiponectin, a key mediator of insulin sensitivity. Population studies have shown an inverse relationship between ferritin and adiponectin,(34) and a positive correlation between increasing ferritin and features of metabolic syndrome.(10) In this study, adiponectin correlated with liver iron, suggesting that

iron may contribute to its regulation. Recently, adipocyte iron was shown to directly influence adiponectin production and insulin sensitivity.(35)

Despite preliminary evidence from human and animal studies implicating iron in the pathogenesis of NAFLD and insulin resistance, two randomized controlled trials and a recent meta-analysis failed to demonstrate any short-term benefit from iron depletion by venesection on insulin resistance and markers of liver damage in patients with NAFLD and/or dysmetabolic iron overload syndrome (DIOS) with mild iron overload.(36-38) The reasons for this are unclear, but may relate to patient selection/study design, or even the location of pathogenic iron deposition in NAFLD being less amenable to venesection. More targeted approaches to hepatic iron redistribution, such as hepcidin antagonism or the upregulation of ferroportin to facilitate iron export from reticulo-endothelial cells, may yet yield therapeutic benefit, but as such remain in experimental phases of development.(39)

In conclusion, this study of two independent cohorts of European NAFLD patients demonstrates that liver iron is mildly increased predominantly in Kupffer cells, and is the major determinant of serum ferritin, a prognostic marker in NAFLD. Ferritin also reflects markers of insulin resistance, such as adiponectin and liver hepatic fat content, and varies according to NAFLD fibrosis stage. These findings suggest a role for disordered iron regulation in the pathogenesis of NAFLD, and therefore a potential therapeutic target.

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Figure legends

Figure 1. Serum ferritin and iron status.

(A and B) Serum ferritin and liver iron (as determined by MRI T2*, with an inverse relationship to liver iron levels) differed significantly between NAFLD patients (n=51), viral hepatitis (n=30) and healthy controls (ANOVA, p=0.0045 and p=0.0025 respectively). (C-E) In NAFLD patients, serum ferritin correlated closely with serum hepcidin (r=0.73, p<0.0001) and liver iron (r=-0.76, p<0.0001), while hepcidin correlated significantly with liver iron also (r=-0.6, p<0.0001). (F) In the liver biopsy validation cohort, a significant positive association between serum ferritin and hepatic iron concentration (HIC) was seen (r=0.28, p<0.0001).

Figure 2. Serum ferritin and metabolic features

(A and B) In NAFLD patients (n=51), significant positive associations between serum ferritin and hepcidin with hepatic lipid content (MR spectroscopy, MRS) were noted ($r=0.57$, $p<0.0001$ and $r=0.42$, $p=0.0024$, respectively). (C and D) Weak negative associations were observed between serum ferritin and hepcidin and serum total adiponectin ($r=-0.27$, $p=0.05$ and $r=-0.28$, $p=0.049$, respectively, Fig 2c and 2d). (E) A significant correlation between serum total adiponectin and liver iron by T2* was demonstrated ($r=0.39$, $p=0.0049$).

Figure 3. Serum ferritin and fibrosis stage

(A-C) Within the NAFLD MR cohort, significant differences in serum ferritin, hepcidin and liver iron (as determined by MRI T2*, with an inverse relationship to liver iron levels) were noted (ANOVA, $p=0.0007$, $p<0.0001$, $p<0.002$, respectively) across the disease fibrosis stages (F0-4). (D and E) Hepatic lipid content increased significantly from F0/1 to F2 fibrosis, while splenic iron levels fell significantly (indicated by increasing T2* values, $p=0.0003$) as fibrosis progressed. (F) In the liver biopsy validation cohort, a similar trend of increasing ferritin in initial stages and decline in cirrhosis was noted ($p<0.05$; F0/1=302, F2=63, F3=20, F4=19).

Table 1. Baseline Characteristics^ (MRI Cohort)		NAFLD (n=51)	Viral Hepatitis (n=30)	Controls (n=20)	p value (ANOVA)
Demographics	**Female** (%)	37.3%	17%	60%	0.01[+]
	Age (yrs)	55 (12.7)	50 (12)	58 (10)	0.08
	Weight (kg)	93.1(20)	75 (16)	73.1 (11)	<0.0001
	BMI (kg/m^2)	31.3 (5)	25.2 (4)	24.5 (2.3)	<0.0001
Biochemistry	**Bilirubin** (µmol/L)	13 (9)	14 (8)	13 (8)	0.58[*]
	ALT (IU/L)	69 (47)	95 (88)	20 (15)	0.09[*]
	AST (IU/L)	50 (30)	59 (37)	25 (7)	0.23[*]
	Platelets (x10^9/L)	216 (72)	181 (72)	248 (42)	0.04[*]
	Glucose (mmol/L)	6 (2.6)	5.1 (1.1)	5.4 (0.4)	0.079
	T. Cholesterol (mmol/L)	4.8 (1.3)	4.2 (1)	5.6 (1)	0.0004
	CRP (mg/L)	5.3 (6.4)	1.5 (3.2)	2.2 (3.4)	0.005
	Insulin (mIU/ml)	330 (299)	114.1 (100)	46.8 (27)	<0.0001
	HOMA-IR	2.45 (2.2)	0.99 (0.8)	0.39 (0.2)	<0.0001
	Total Adiponectin (µg/ml)	5.97 (3.2)	n/a	11.5 (1.3)	<0.0001
	Leptin (ng/ml)	25.5 (17)	5.5 (4.9)	12 (11.5)	<0.0001
Stage	**MRI Hepatic lipid content**	15.2 (12)	2.0 (2)	2.3 (2.6)	<0.0001
	Fibroscan (kPa)	12.9 (13.8)	12.1 (10.5)	4.2 (0.9)	0.81[*]
	Fibrosis stage (n, %) • Mild • Moderate • Severe	17 (33.3%) 16 (31.4%) 18 (35.3%)	7 (23%) 17 (57%) 6 (20%)	n/a	0.72

^Mean+/- standard deviation, unless specified. [+]Chi-squared test. *Unpaired t-test of NAFLD vs. Viral hepatitis

[$]Brunt for NAFLD, Ishak for Viral hepatitis. Mild fibrosis equates to Brunt 0-1 or Ishak 0-1, moderate fibrosis to Brunt 2 or Ishak 2-4 and severe fibrosis to Brunt 3-4 or Ishak 5-6

BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance.

Table 2. Markers of iron status^ (MRI Cohort)	NAFLD (n=51)	HCV (n=22)	HBV (n=8)	Controls (n=20)	p value (ANOVA)
Biochemistry					
Serum iron (µmol/L)	16.4 (6.9)	22.5 (10.6)	17 (7.6)	17.5 (5.3)	0.04
Serum ferritin (µg/L) *	137 (1944)	215 (783)	187 (482)	56.9 (405)	0.0058$
Transferrin Sat (%)	23.7 (11)	29.5 (14)	26 (13)	31.9 (8.6)	0.018
Hepcidin (ng/ml) *	57.3 (379)	45.3 (165)	52.2 (78)	61.9 (201)	0.06$
Hepcidin:Ferritin ratio	0.82 (0.17)	0.77 (0.3)	0.81 (0.3)	1.1 (0.2)	0.20
Liver histology					
Prussian blue staining • Positive (n,%) • Grade (0-4)	 7 (14%) 1	 3 (14%) 1	 0	 -	 -
Staining pattern • Hepatocellular • Reticuloendothelial • Mixed	 2 3 2	 1 1 1	-	-	-
MRI					
Liver T2*lateral (ms)	16.4 (5.5)	20.3 (5.4)	19 (5.6)	20.2 (3.5)	0.0025
Spleen T2* (ms)	27.9 (16.6)	35.4 (20.5)	24.3 (13.5)	34.5 (13.6)	0.29

^Mean+/- standard deviation unless indicated. *median (range) $Kruskal-Wallis test

Table 3. Univariate and multivariate analysis of variables associated with serum ferritin in NAFLD MRI Cohort

Variable	Beta	p value	Multivariate	Beta	p value
HLC	0.56	<0.0001		-	-
T2*Liver	-.757	<0.0001		-0.492	0.001
T2Spleen	-.302	0.031		-	-
Hb	0.465	0.001		-	-
WCC	0.289	0.040		-	-
ALT	0.355	0.011		-	-
Glucose	-0.287	0.041		-	-
Adiponectin	-0.273	0.053		-	-
Hepcidin (log)	0.726	<0.0001		0.315	0.004
GDF15	-0.365	0.009		-	-
A model combining T2* liver, hepatic lipid content (HLC), haemoglobin (Hb), alanine aminotransferase (ALT) and hepcidin yielded an adjusted R^2 value of 0.67, thereby accounting for 67% of the variance in serum ferritin levels.					

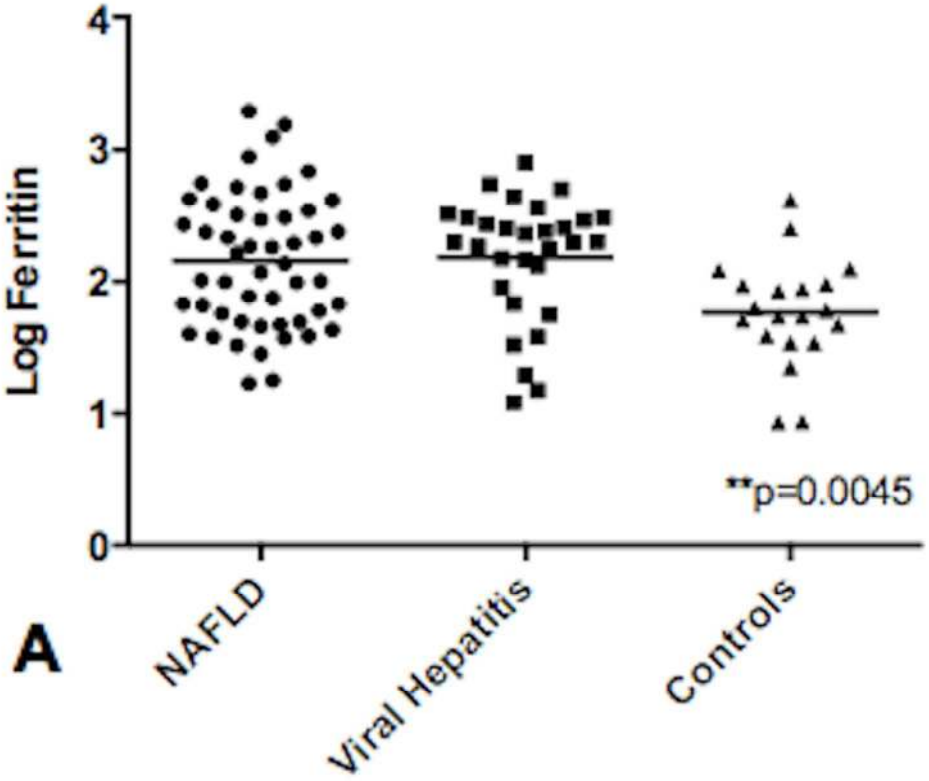

Figure 1A

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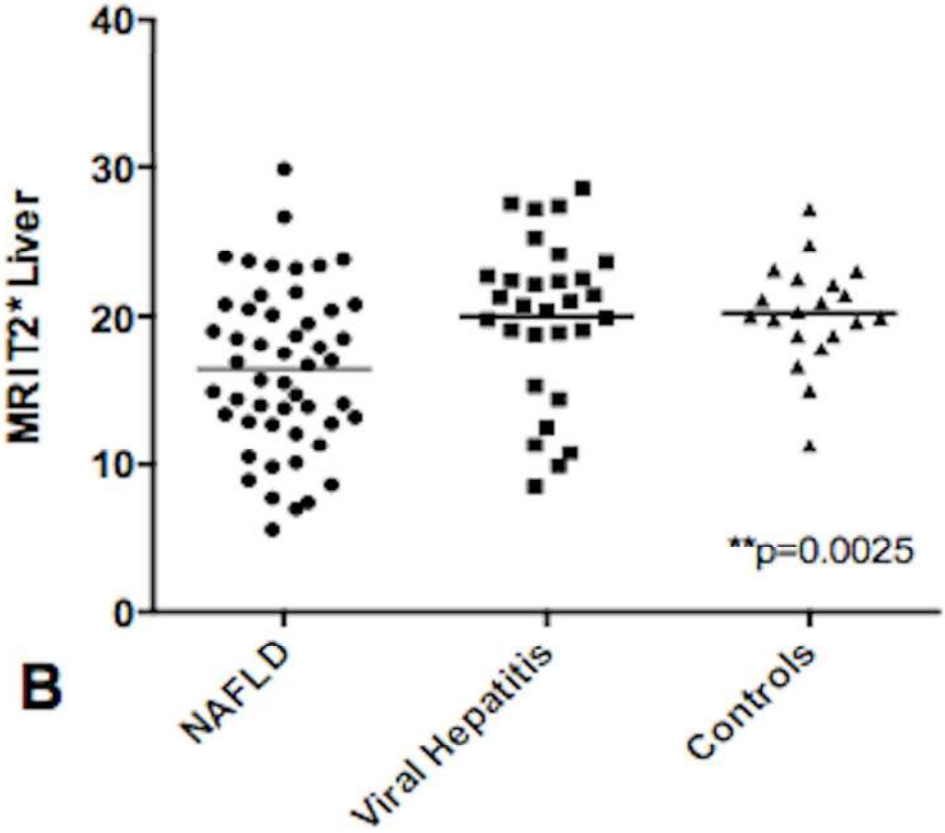

Figure 1B

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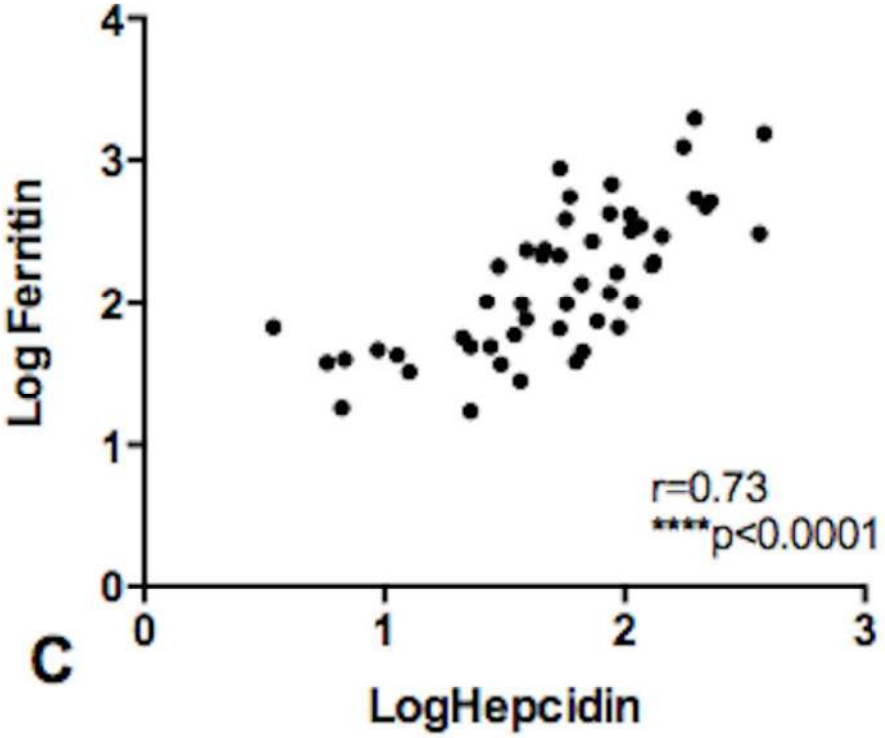

Figure 1C

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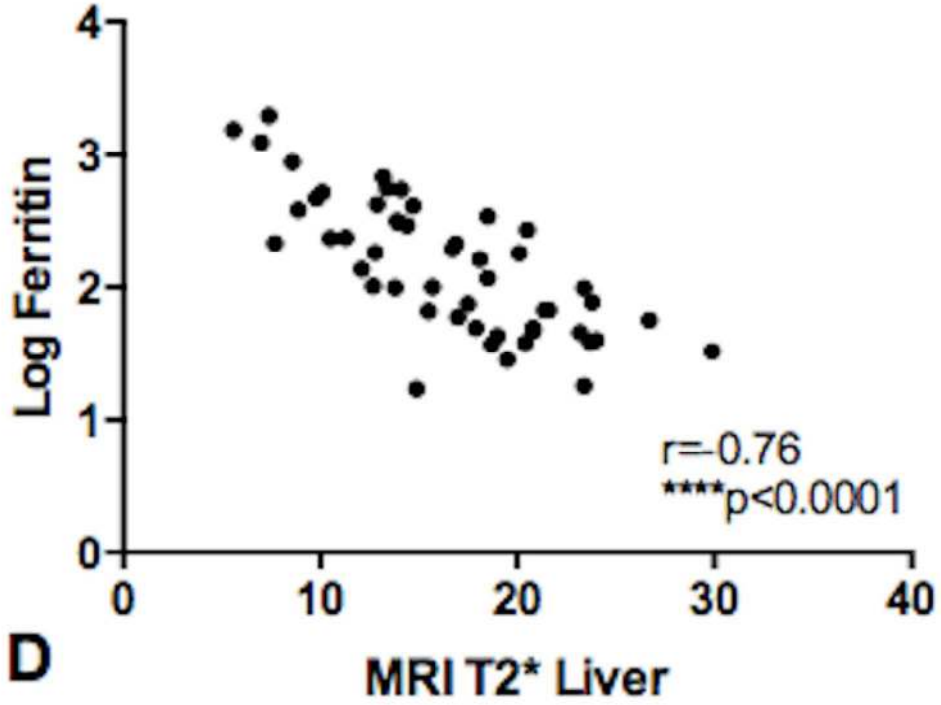

Figure 1D

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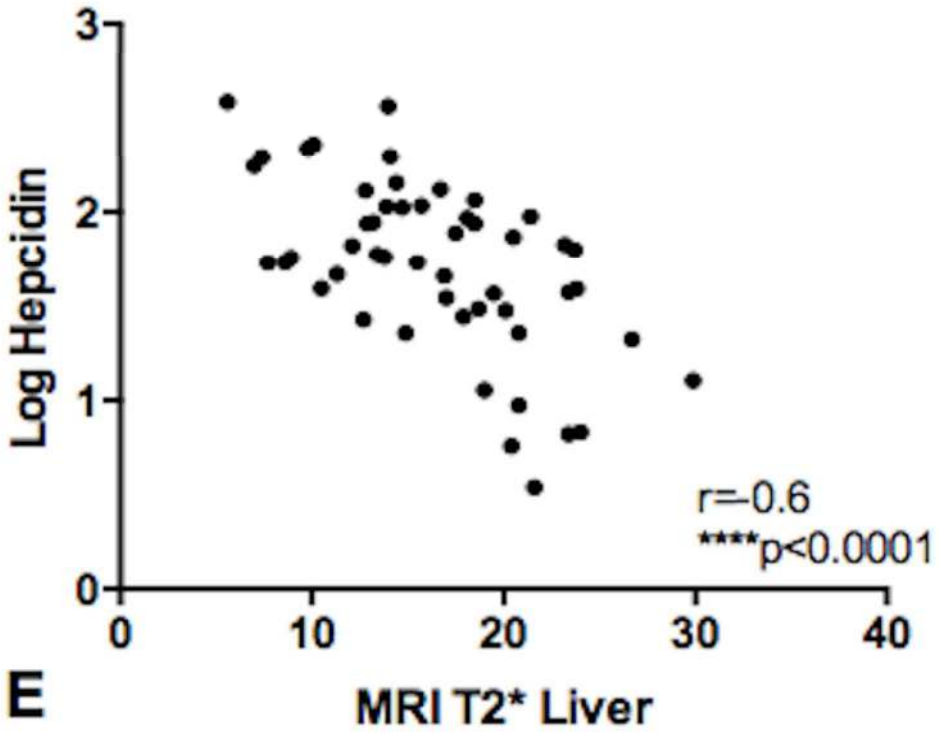

Figure 1E

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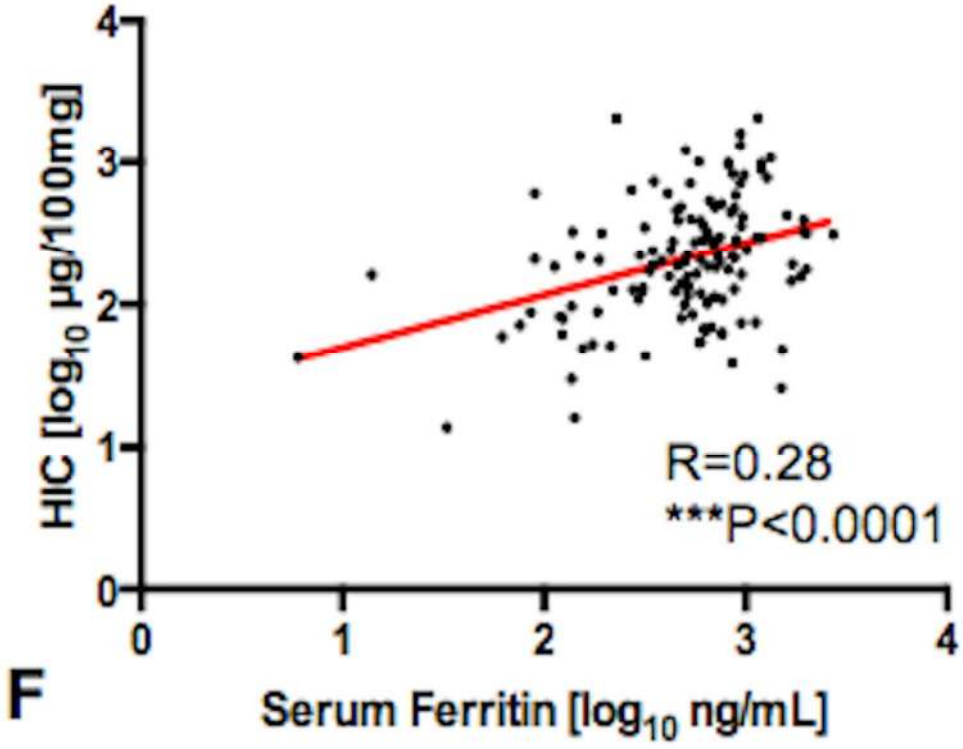

Figure 1F

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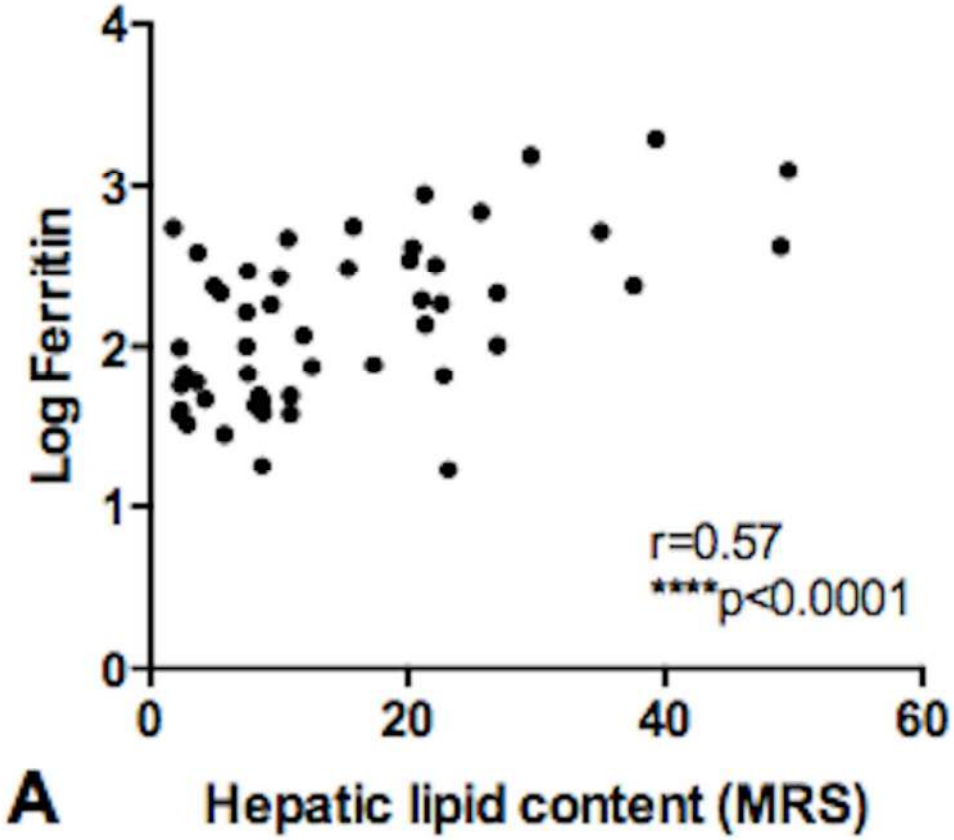

Figure 2A

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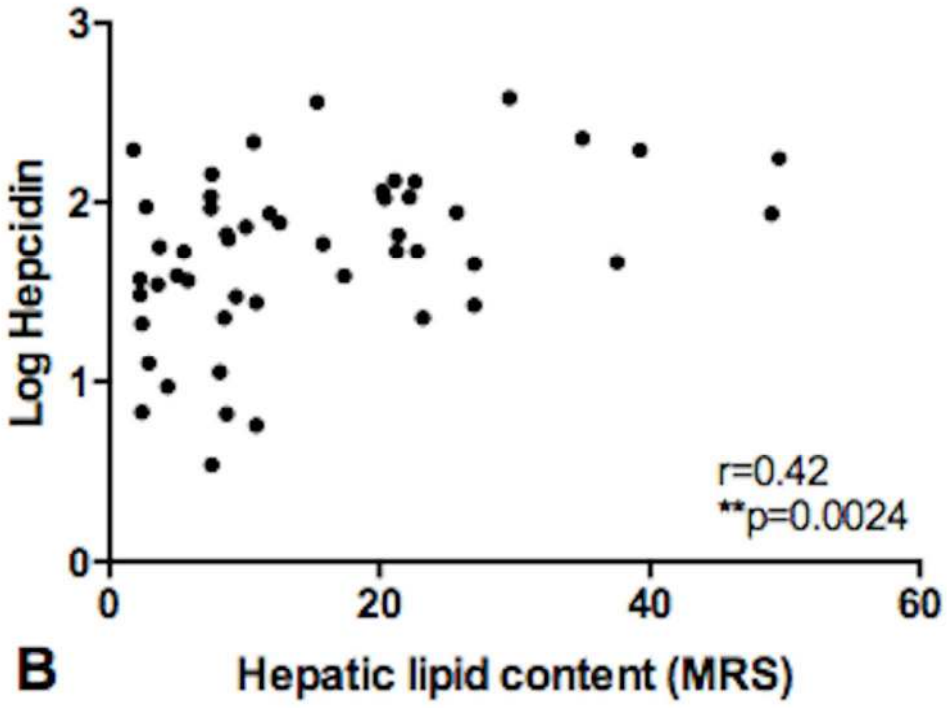

Figure 2B

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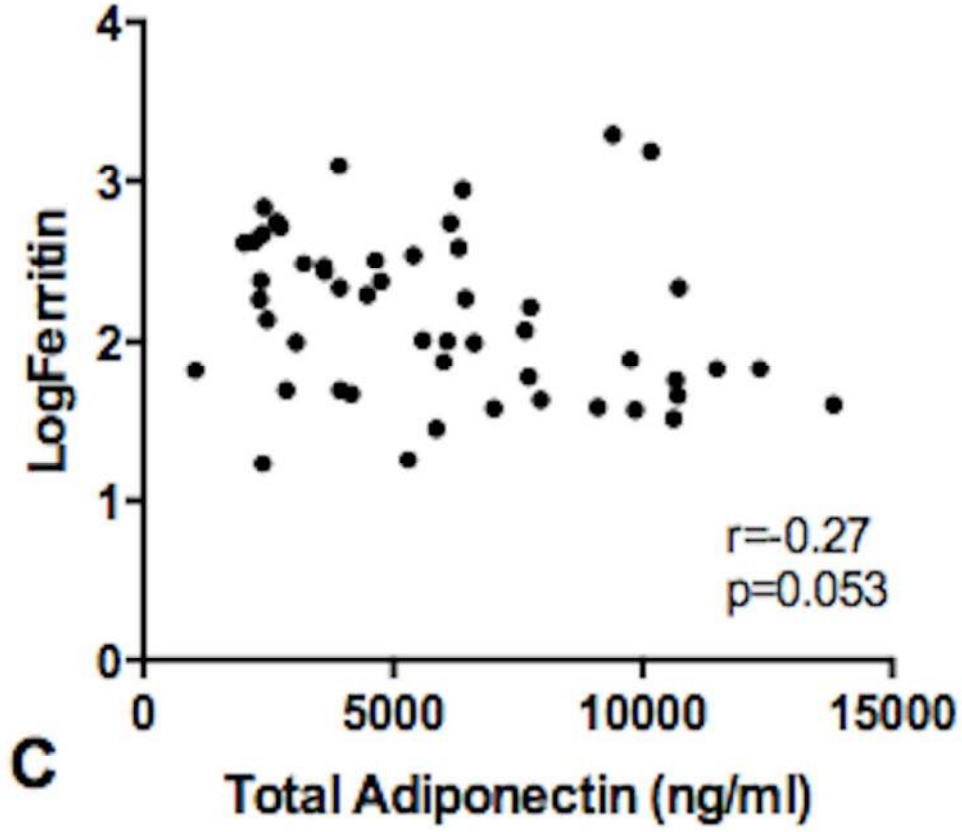
C
LogFerritin
4
3
2
1
0
0
5000
10000
15000
Total Adiponectin (ng/ml)
r=-0.27
p=0.053

Figure 2C

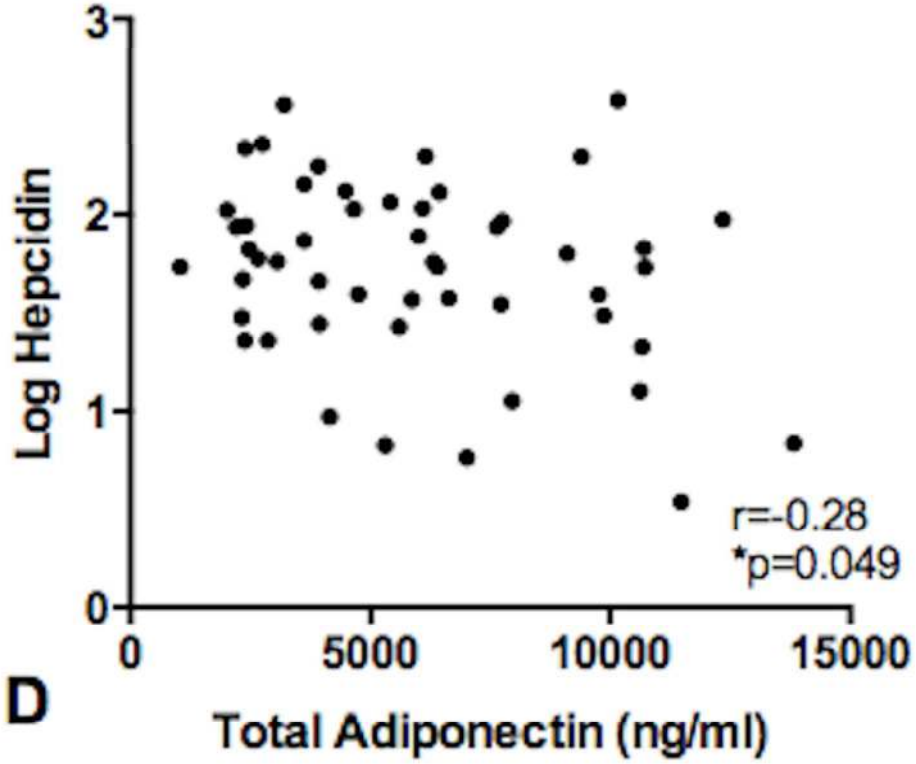

Figure 2D

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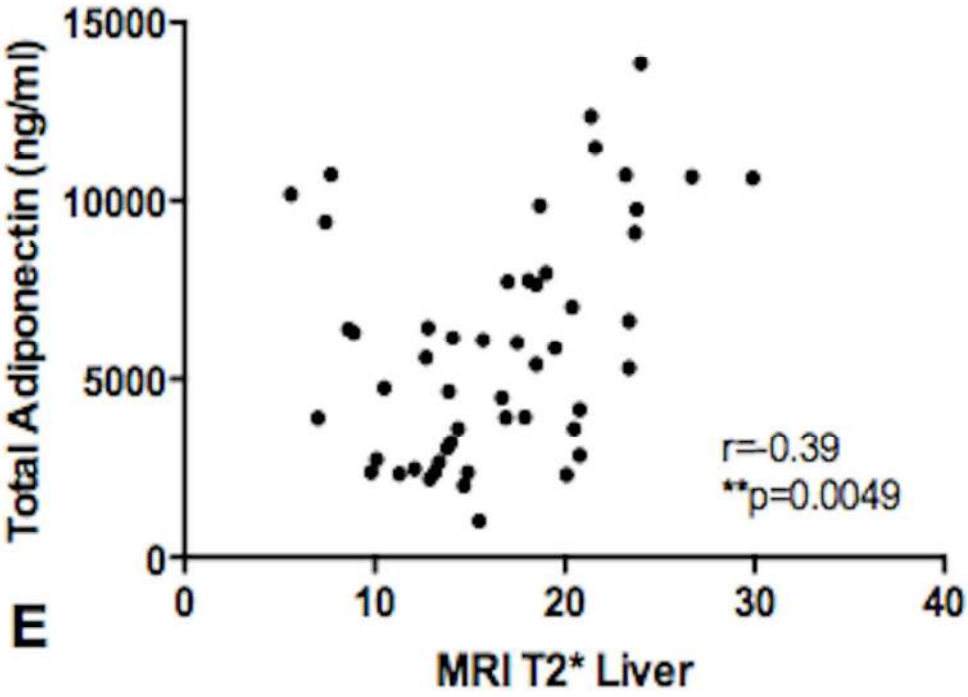

Figure 2E

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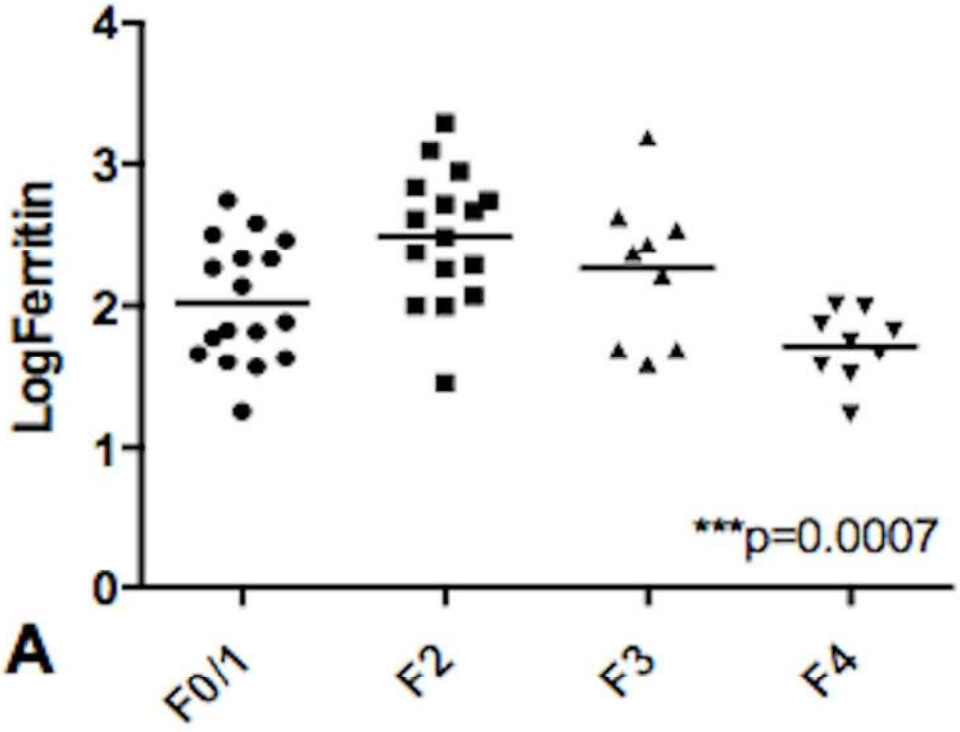

Figure 3A

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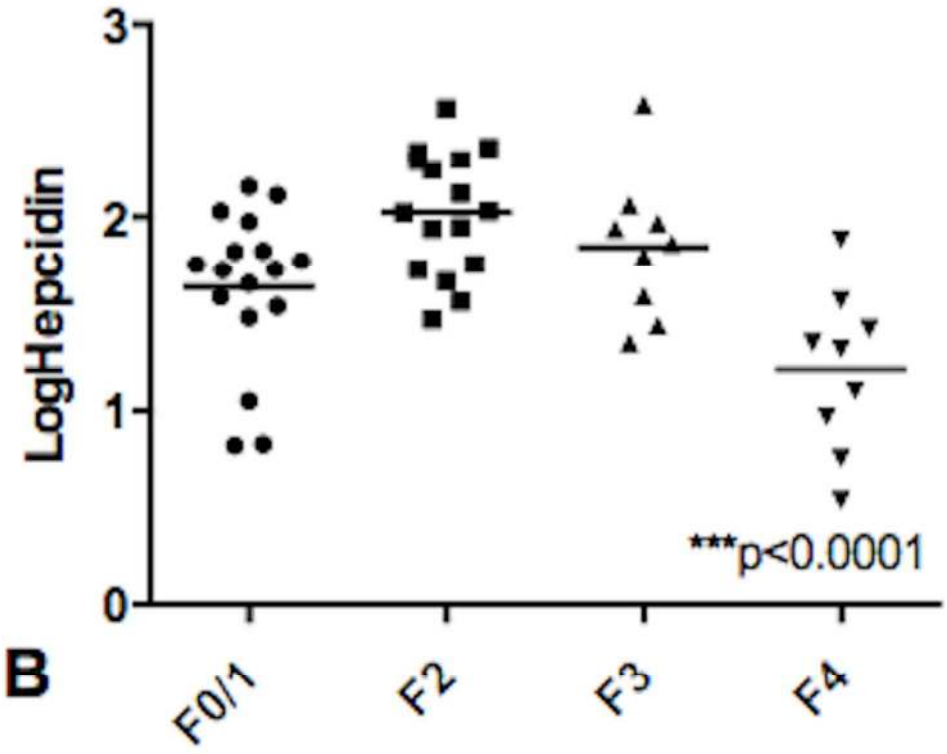

Figure 3B

98x80mm (300 x 300 DPI)

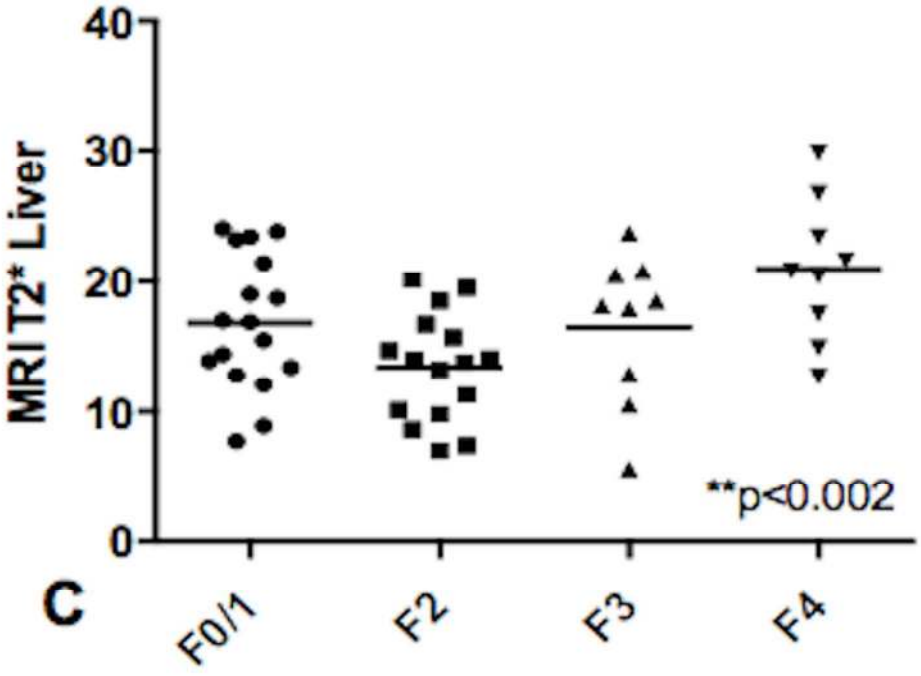

Figure 3C

107x79mm (300 x 300 DPI)

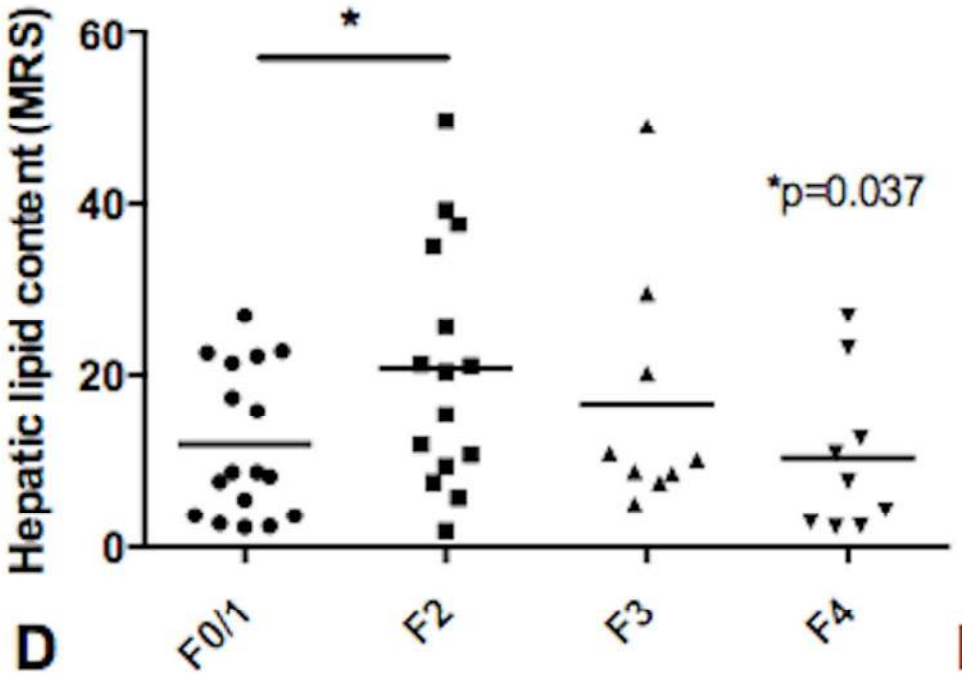

Figure 3D

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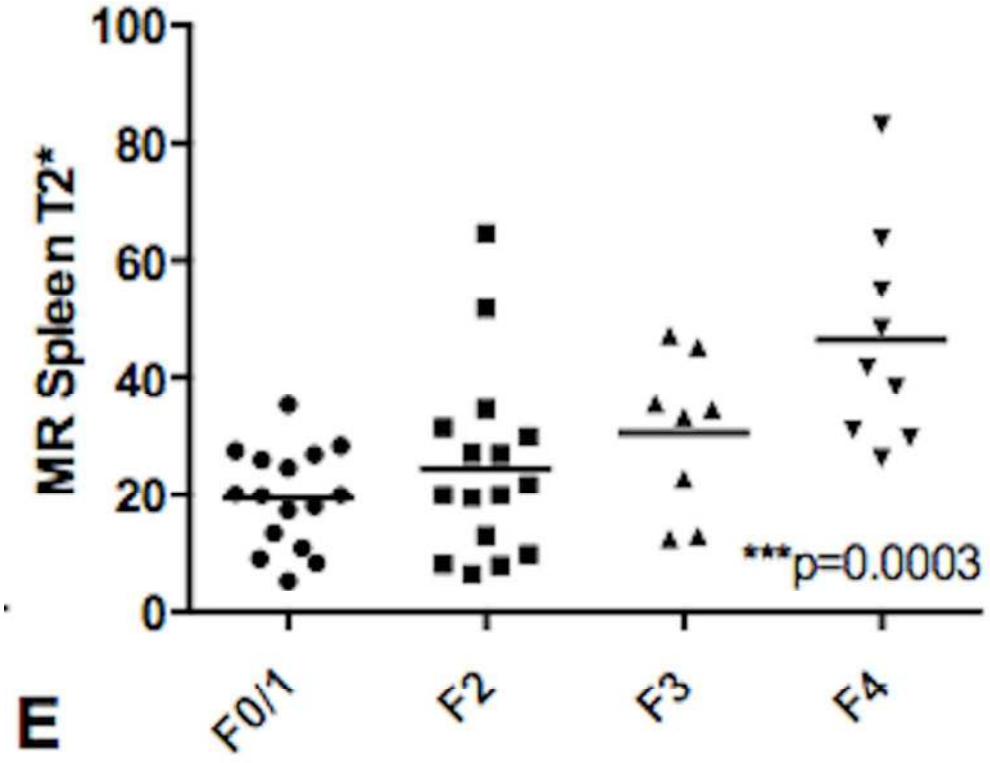

Figure 3E

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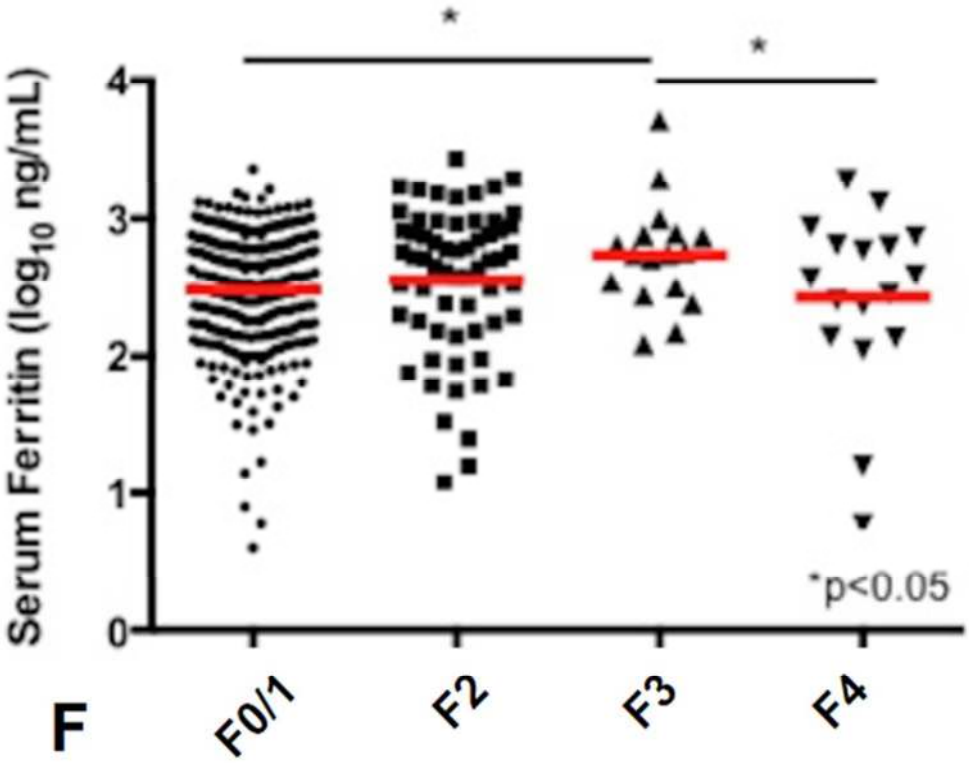

Figure 3F

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