Serum Ferritin Is an Independent Predictor of Histologic Severity and Advanced Fibrosis in Patients With Nonalcoholic Fatty Liver Disease

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Serum ferritin (SF) levels are commonly elevated in patients with nonalcoholic fatty liver disease (NAFLD) because of systemic inflammation, increased iron stores, or both. The aim of this study was to examine the relationship between elevated SF and NAFLD severity. Demographic, clinical, histologic, laboratory, and anthropometric data were analyzed in 628 adult patients with NAFLD (age, ≥18 years) with biopsy-proven NAFLD and an SF measurement within 6 months of their liver biopsy. A threshold SF >1.5 × upper limit of normal (ULN) (i.e., >300 ng/mL in women and >450 ng/mL in men) was significantly associated with male sex, elevated serum alanine aminotransferase, aspartate aminotransferase, iron, transferrin-iron saturation, iron stain grade, and decreased platelets ($P < 0.01$). Histologic features of NAFLD were more severe among patients with SF >1.5 × ULN, including steatosis, fibrosis, hepatocellular ballooning, and diagnosis of NASH ($P < 0.026$). On multiple regression analysis, SF >1.5 × ULN was independently associated with advanced hepatic fibrosis (odds ratio [OR], 1.66; 95% confidence interval [CI], 1.05-2.62; $P = 0.028$) and increased NAFLD Activity Score (NAS) (OR, 1.99; 95% CI, 1.06-3.75; $P = 0.033$). *Conclusions:* A SF >1.5 × ULN is associated with hepatic iron deposition, a diagnosis of NASH, and worsened histologic activity and is an independent predictor of advanced hepatic fibrosis among patients with NAFLD. Furthermore, elevated SF is independently associated with higher NAS, even among patients without hepatic iron deposition. We conclude that SF is useful to identify NAFLD patients at risk for NASH and advanced fibrosis. (HEPATOLOGY 2012;55:77-85)

Nonalcoholic fatty liver disease (NAFLD) is now recognized as the most common cause of liver disease and may be present in up to 20% of the U.S. population.[1] Expression of ferritin, the primary tissue iron-storage protein in the liver, where most extra body iron is stored, is induced in primary or secondary iron overload disorders, resulting in increased hepatic and circulating ferritin levels.[2] However, ferritin is also an acute-phase protein and can also be induced in the setting of systemic inflammation.[3,4] Hyperferritinemia has been previously observed in obesity-related chronic inflammatory conditions, such as diabetes and metabolic syndrome.[5-8] We and others have previously reported that serum ferritin (SF) may be increased in the general population in relationship to alcohol consumption, as well as in chronic liver disease due to hepatitis C and alcohol.[9-16] In such conditions, SF elevation may or may not be

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; ESLD, end-stage liver disease; HC, hepatocellular; IL, interleukin; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis; NFκB, nuclear factor kappa light-chain enhancer of activated B cells; OR, odds ratio; RES, reticuloendothelial system; SF, serum ferritin; TIBC, total iron-binding capacity; TNF-α, tumor necrosis factor alpha; TS, transferrin-iron saturation; ULN, upper limit of normal.

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accompanied by increased hepatic iron deposition.[10-12,15,16] A number of *in vitro* and *in vivo* studies in hepatocytes and liver tissue suggest that inflammatory stimuli, particularly the proinflammatory cytokine, tumor necrosis factor alpha (TNF-α), up-regulates ferritin.[17-23] This effect is additive upon the addition of iron.[24] Oxidative stress may also up-regulate ferritin, depending on the specific oxidant stimuli[25-29] at the level of transcription and translation.[24,25,27,30] Therefore, it is plausible that SF may reflect increased disease severity in NAFLD either because of increased ongoing hepatic or systemic inflammation or increased body iron stores or a combination of these factors. Previous studies examining the relationship between SF level and histologic severity in NAFLD have found conflicting results, with some investigators reporting that SF levels are associated with increased histologic severity and the presence of NASH, whereas others have not.[31-43] We have previously described that patients with NASH have significantly higher median SF levels, compared to those with NAFLD.[44] The aims of the current study were to examine the relationship of SF to clinical, histologic, laboratory, and anthropometric data in adult patients with NAFLD enrolled in the Nonalcoholic Steatohepatitis Clinical Research Network (NASH CRN), identify whether specific threshold levels of SF would identify patients with more advanced disease, and examine the relationship between SF level, hepatic iron stores, and histologic features among these subjects.

Patients and Methods

A total of 1,635 subjects were enrolled in the NASH CRN Database, PIVENS, and TONIC studies between 2004 and 2008 per inclusion criteria described elsewhere.[44-46] Of these, subjects that did not meet the following criteria were excluded: (1) age less than 18 years at time of biopsy (368 subjects); (2) lack of liver biopsy sample for central pathology review by the NASH CRN Pathology Committee (182 subjects); (3) SF value not available within 6 months of biopsy (418 subjects); and (4) lack of NAFLD diagnosis because of less than 5% steatosis (39 subjects). The remaining 628 subjects constituted our study group. Patients with known hemochromatosis (defined as the presence of hepatic iron index [hepatic iron (μmol/g)/age(years)] $\geq$1.9 or removal of more than 4 g of iron by phlebotomy), C282Y homozygosity for the *HFE* gene, or unexplained substantial hepatic iron overload ($\geq$3+ stainable iron on liver biopsy) were excluded from all NASH CRN studies. Demographic information, such as age, sex, ethnicity, and race and medical history to identify comorbidities and medications, were obtained from patient interviews during screening. A physical exam, including body-weight and height measures, was performed. Laboratory data, including hepatic, hematologic, metabolic, lipid, and serum iron assessments, were analyzed for subjects with values collected within 6 months of liver biopsy. Total dietary consumption of iron, vitamin C, tea, and coffee were determined from the Block 98 food frequency questionnaire (NutritionQuest, Berkeley, CA), and alcohol consumption was determined from the Alcohol Use Disorders Identification Test (AUDIT-C) questionnaires completed during study visits closest to the time of biopsy.[47] In addition, 552 of the 628 (88%) subjects included in this study had available hepatic iron staining, which was routinely evaluated as part of the liver histologic assessment performed centrally by the NASH CRN Pathology Committee.

Histological Assessment. Histologic features of NAFLD and iron accumulation were assessed by the Pathology Committee of the NASH CRN in a centralized consensus review format. Pathologists were blinded to all clinical, laboratory, and demographic information. NAFLD features were scored as previously described.[48] Semiquantitative grading and pattern of hepatic iron staining, using Perls' iron stain, was scored by the Pathology Committee, as previously described.[49]

Statistical Analysis. The relationship between SF and variables of interest was examined with SF as a dichotomous variable, defined *a priori* to be practical in a clinical setting; $\leq$1.5 × upper limit of normal (ULN) versus >1.5 × ULN (ULN was defined as <200 ng/mL in women and <300 ng/mL in men; values were adopted from the Hemochromatosis and Iron Overload Screening Study).[50] Baseline demographic, clinical, and laboratory characteristics were recorded as number and percentage for categorical data and means and standard deviation or medians and interquartile range for continuous data. Continuous variables, including laboratory measures that were not normally

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distributed, were analyzed using the Wilcoxon rank-sum test. Categorical variables, including histological features such as fibrosis stage, steatosis, and lobular inflammation grade, were analyzed using either Fisher's exact or chi-square tests, where appropriate. Stepwise forward multivariate logistic regression was used to examine the relationship between threshold SF levels SF>ULN, SF >1.5 × ULN, and SF >2.5 × ULN and the presence of advanced fibrosis after controlling for the effect of the following variables selected *a priori*: age at biopsy, sex, presence of diabetes, body mass index (BMI), and alanine aminotransferase (ALT). Stepwise ordinal regression was used to examine the relationship between threshold SF >1.5 × ULN levels and NAFLD Activity Score (NAS) after controlling for the above covariates, separately among patients with each of the three distinct hepatic iron deposition patterns.[49] Variables not independently associated with the dependant variable, using a threshold of $P \leq 0.20$, were excluded from all models. All analyses were performed using Stata software (version 9; StataCorp., College Station, TX). Nominal, two-sided *P* values were used and were considered to be statistically significant if $P < 0.05$; no adjustments for multiple comparisons were made.

Results

Patient Characteristics. A total of 628 NASH CRN adult subjects (age ≥18 years) with biopsy-proven NAFLD (defined as >5% steatosis) and a SF measurement within 6 months of their liver biopsy were evaluated in the present study. The distribution of SF values for the study cohort is presented graphically in Fig. 1. Subjects were divided according to sex and threshold SF value of 1.5 × ULN (i.e., >300 ng/mL in women and >450 ng/mL in men). The majority of women tended to be in the lower range of this threshold (see Fig. 1B,D). In contrast, males were more evenly distributed throughout each threshold group (see Fig. 1A,C). More than half of all subjects (324 of 628) were women with SF ≤1.5 × ULN. A comparison of clinical and demographic data for subjects with SF ≤1.5 × ULN to those with SF >1.5 × ULN is shown in Table 1. Approximately 20% (128 of 628) of the study population had SF values >1.5 × ULN. There were 84 subjects (13%) who had SF>ULN, but ≤1.5 × ULN (data not shown). The majority of patients with SF >1.5 × ULN were female (54%); however, a greater proportion of males had SF >1.5 × ULN, compared to females (25% versus 18%; $P = 0.02$). Increased SF was associated with lower BMI ($P = 0.004$). There were no other significant differences in age at biopsy, race or ethnicity, or the presence of diabetes or metabolic syndrome between normal and elevated SF groups. The proportion of subjects carrying *HFE* mutations was not significantly different between SF categories, including SF >2.5 × ULN (Table 1 and data not shown).

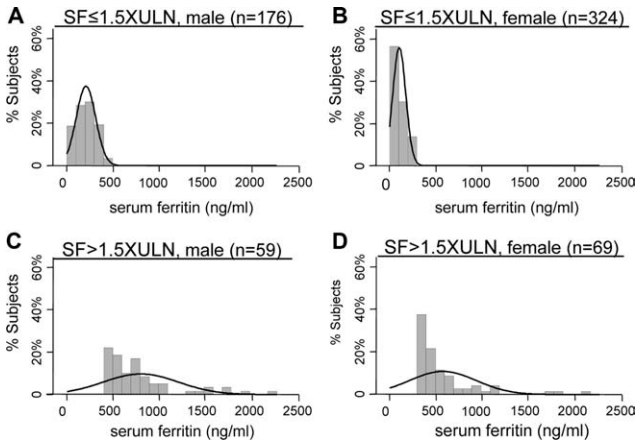

Fig. 1. Histogram of distribution of SF values according to sex, above and below the threshold SF >1.5 × ULN. The relative proportion of subjects by SF values are shown for each of the four categories: (A) SF ≤1.5 × ULN, male; (B) SF ≤1.5 × ULN, female; (C) SF >1.5 × ULN, male; and (D) SF >1.5 × ULN, female. Each bin corresponds to a range of SF values of 100 ng/mL. The density estimation plot is shown by the smooth line.

Relationship Between Laboratory Data and SF Level. Differences in laboratory tests between patients with SF ≤1.5 × ULN versus SF >1.5 × ULN are shown in Table 2. Patients with SF >1.5 × ULN had higher serum aspartate aminotransferase (AST), ALT, gamma-glutamyl transferase, and direct bilirubin and lower platelet count ($P < 0.02$). In contrast, metabolic abnormalities, including fasting insulin, glucose, homeostasis model assessment of insulin resistance, and lipid levels, were not different between groups (data not shown). As was expected, patients with hyperferritinemia had higher serum iron studies, including iron, ferritin, and percent transferrin-iron saturation (TS), and lower total iron-binding capacity (TIBC) ($P < 0.0001$).

Relationship Between SF Level and Histologic Severity of NAFLD. Increased histologic severity of NAFLD was associated with higher SF values (Table 3). A higher proportion of patients with SF >1.5 × ULN showed increased severity of steatosis, lobular inflammation, hepatocellular (HC) ballooning, and fibrosis, compared to patients with SF ≤1.5 × ULN ($P \leq 0.026$). Subjects with SF >1.5 × ULN were also more likely to have a definite diagnosis of NASH than those with SF ≤1.5 × ULN (70% versus 59%; $P = 0.017$). The proportion of subjects with advanced (i.e., stage 3 or 4) fibrosis at higher SF levels was 32% of SF >1.5 × ULN, 42% SF >2.5 × ULN, and 34% SF >3.5 ×

Table 1. Characteristics of Patients Above and Below SF 1.5 × ULN

Characteristic*	SF ≤1.5 × ULN (N = 500)	SF >1.5 × ULN (N = 128)	Total (N = 628)	P Value†
Sex, n (%)				0.02
Male	176 (75)	59 (25)	235 (37)	
Female	324 (82)	69 (18)	393 (63)	
Age, mean (years)	47.7 ± 11.8	47.4 ± 11.8	47.7 ± 11.8	0.14
Race, n (%)				0.14
White	404 (79)	106 (21)	510 (81)	
Black	14 (93)	1 (7)	15 (2)	
Asian	17 (65)	9 (35)	26 (4)	
American Indian/Alaskan Native	17 (77)	5 (23)	22 (4)	
Other	48 (87)	7 (13)	55 (9)	
Ethnicity, n (%)				0.30
Non-Hispanic	432 (79)	115 (21)	547 (87)	
Hispanic	68 (84)	13 (16)	81 (13)	
BMI, mean (kg/m^2)	34.4 ± 6.5	32.6 ± 5.5	34.0 ± 6.3	0.004
BMI category, n (%)				0.01
<25 (kg/m^2)	23 (79)	6 (21)	23 (1.3)	
25<30 (kg/m^2)	110 (71)	45 (29)	148 (23)	
≥30 (kg/m^2)	366 (83)	77 (17)	462 (73)	
Waist-hip ratio	0.93 ± 0.08	0.94 ± 0.07	0.93 ± 0.07	0.15
Diabetes diagnosis, n (%)	128 (78)	36 (22)	164 (26)	0.56
HFE genotype‡				
WT/WT	244 (81)	57 (19)	301 (61)	Reference
C282Y/WT	40 (77)	12 (23)	52 (11)	0.49
H63D/WT	99 (76)	31 (24)	124 (25)	0.25
H63D/H63D	6 (67)	3 (33)	9 (1.8)	0.38
C282Y/H63D	3 (50)	3 (50)	6 (1.2)	0.09
Metabolic syndrome, n (%)	344 (80)	88 (20)	432 (69)	0.99
No. of alcoholic drinks/week	0.6 ± 1.3	0.8 ± 1.7	0.6 ± 1.4	0.67
No. of coffee/tea drinks/day	1.7 ± 1.5	1.5 ± 1.4	1.7 ± 1.5	0.27
Dietary iron consumed (mg/day)	14 ± 8	14 ± 8	14 ± 8	0.64
Dietary vitamin C (mg/day)	107 ± 82	98 ± 72	105 ± 80	0.40

Abbreviations: SF, serum ferritin; BMI, body mass index; WT, wild type; ULN, serum ferritin upper limit of normal (defined as 200 mg/dL in females and 300 mg/dL in males).

*Values are number and percentage or mean ± standard deviation.

†*P* values from chi square or Fisher's exact test for categorical variables; Wilcoxon rank-sum test for continuous variables.

‡*HFE* genotype available in 492 subjects; *P* values compared to WT/WT.

ULN. To investigate whether various threshold levels of elevated SF would be independently associated with advanced fibrosis, we used a multivariate stepwise logistic regression analysis, including the following variables, selected *a priori*, known to be associated with severity of NAFLD: age at biopsy, sex, BMI, presence of diabetes, and ALT. Three separate models were created using SF as a dichotomous independent variable, as follows: (1) SF >ULN; (2) SF >1.5 × ULN; and (3) SF >2.5 × ULN. There was a progressive trend toward an independent association between increasing SF level and advanced hepatic fibrosis (Table 4). Both SF >1.5 × ULN and >2.5 × ULN were independent predictors of advanced fibrosis (odds ratio [OR], 1.67; $P = 0.028$; OR, 2.46; $P = 0.005$, respectively) using a logistic regression modeling controlling for BMI, age, sex, type 2 diabetes, and serum ALT. In all three models, age, BMI, and presence of diabetes were the only other variables independently associated with advanced hepatic fibrosis ($P < 0.002$).

Relationship of SF and Hepatic Iron Deposition Pattern. As described above, patients with elevated SF were also more likely to have increased serum iron and TS, compared to those with SF≤ULN. Therefore, it is possible that increased hepatic iron loading may be responsible for the increased severity of liver disease among patients with elevated SF. We have previously shown that the presence and pattern of stainable hepatic iron was associated with advanced histologic features among patients with NASH; in addition, NAFLD patients with reticuloendothelial system (RES) iron deposition were more likely to have NASH and advanced fibrosis, whereas those with HC iron were more likely to have milder disease.[49] We therefore examined the relationship between SF level and stainable hepatic iron in the current cohort using ordinal regression. SF >1.5 × ULN was significantly associated with greater iron content in both RES (OR, 2.22; 95% confidence interval [CI], 1.78-2.76; $P <$

Table 2. Laboratory Value Differences Among Patients Above and Below SF 1.5 × ULN

Variable*	SF ≤1.5 × ULN (N = 500)	SF >1.5 × ULN (N = 128)	*P* Value†
AST (U/L)	43 (31-62)	58 (35-85)	<0.0001
ALT (U/L)	58 (38-85)	81 (53-132)	<0.0001
Gamma-glutamyl transferase (U/L)	43 (28-77)	59 (37-114)	0.0001
Direct bilirubin (mg/dL)	0.10 (0.10-0.20)	0.10 (0.10-0.20)	0.02
Platelets (K/cmm)	246 (203-287)	230 (195-273)	0.015
Hemoglobin (g/dL)	14.3 (13.4-15.2)	15.0 (13.8- 15.7)	0.0001
Serum iron (μg/dL)	84 (65-106)	106 (80-128)	<0.0001
Total iron-binding capacity (μg/dL)	371 (331-417)	345 (301-388)	<0.0001
Transferrin saturation (iron/TIBC)	0.22 (0.17-0.29)	0.29 (0.23-0.41)	<0.0001
Serum ferritin (ng/mL)	121 (71-216)	547 (417-777)	<0.0001

Abbreviations: SF, serum ferritin; ULN, upper limit of normal; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TIBC, total iron-binding capacity.
*Values are medians (interquartile range).
†*P* values from Wilcoxon rank-sum test.

0.001) or HC cells (OR, 1.5; 95% CI, 1.03-1.91; $P < 0.001$), compared to those with lower SF levels. To determine whether SF could identify patients with more advanced disease, even in the absence of hepatic iron loading, we compared the NAS separately among patients with each of the three distinct hepatic iron deposition patterns using ordinal regression modeling after controlling for covariates BMI, age, sex, type 2 diabetes, and serum ALT. Shown in Fig. 2 is a comparison of the mean differences of NAS for subjects above and below SF 1.5 × ULN according to each hepatic iron deposition pattern: HC only, RES only, and mixed HC/RES. SF >1.5 × ULN was independently associated with increased NAS in both subjects without hepatic iron deposits (OR, 1.99; 95% CI, 1.06-3.75; $P = 0.033$) and in subjects with RES iron stain only (OR, 4.59; 95% CI, 1.54-13.65; $P = 0.006$).

Table 3. Histologic Features Among Subjects Above and Below SF 1.5 × ULN

Characteristic	SF ≤1.5 × ULN (N = 500)	SF >1.5 × ULN (N = 128)	*P* Value*
Steatosis grade, n (%)			<0.001
5-33	215 (43)	37 (29)	
34-66	180 (36)	34 (27)	
>66	105 (21)	57 (45)	
Lobular inflammation, n (%)			0.026
<2 under 20x	266 (53)	53 (41)	
2-4 under 20x	184 (37)	54 (42)	
>4 under 20x	50 (10)	21 (16)	
Hepatocellular ballooning, n (%)			0.004
None	169 (34)	30 (23)	
Mild	129 (26)	32 (25)	
More than mild	202 (40)	66 (52)	
Fibrosis stage,† n (%)			<0.001
None	141 (28)	19 (15)	
Mild to moderate, zone 3, perisinusoidal, or portal/periportal only	151 (30)	31 (24)	
Zone 3 and periportal, any combination	85 (17)	36 (28)	
Bridging	78 (16)	34 (27)	
Cirrhosis	42 (8)	7 (6)	
NASH diagnosis, n (%)			0.013
No NASH	111 (22)	14 (11)	
Suspicious/borderline	95 (19)	24 (19)	
Definite	294 (59)	90 (70)	

Values are number and percentage.
Abbreviations: SF, serum ferritin; ULN, upper limit of normal; NASH, nonalcoholic steatohepatitis.
**P* values from chi-square or Fisher's exact test.
†Four subjects were not scored for fibrosis.

Discussion

We have shown, in the present study, that SF has value as a noninvasive marker in identifying patients with NAFLD who are at increased risk of more advanced disease, as indicated by a higher NAS, even among patients without hepatic iron deposition. Furthermore, we have found that SF >1.5 × ULN is

Table 4. Independent Predictors of Advanced Fibrosis on Multivariate Logistic Regression Modeling Using Different SF ULN Levels

	OR	95% CI	P Value
Model 1 (SF >ULN)			
Diabetes present	1.94	1.29-2.90	0.001
Age, years	1.05	1.03-1.07	<0.001
BMI, mean (kg/m^2)	1.05	1.02-1.08	0.002
Model 2 (SF >1.5 × ULN)			
Diabetes present	1.90	1.27-2.85	0.002
SF >1.5 × ULN	1.67	1.06-2.85	0.028
Age, years	1.05	1.03-1.07	<0.001
BMI mean (kg/m^2)	1.05	1.02-1.08	0.001
Model 3 (SF >2.5 × ULN)			
SF >2.5 × ULN	2.46	1.30-4.64	0.005
Diabetes present	1.90	1.27-2.85	0.002
Age, years	1.05	1.03-1.07	<0.001
BMI, mean (kg/m^2)	1.05	1.02-1.08	0.001

Each SF ULN cutoff was modeled individually, including potential confounding variables age, sex, BMI, ALT, and presence of diabetes using stepwise forward multivariate logistic regression. Variables not independently associated with the advanced fibrosis on univariate analysis using a threshold value of $P \leq 0.20$ were excluded from all models.
Abbreviations: SF, serum ferritin; ULN, upper limit of normal; OR, odds ratio; CI, confidence interval; BMI, body mass index.

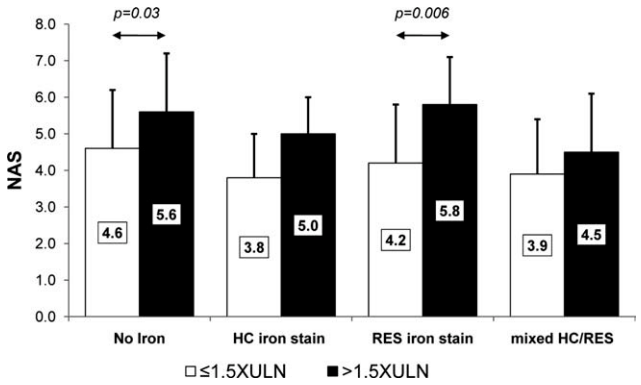

Fig. 2. Relationship between NAS, level of SF, and presence and pattern of iron staining among subjects. Mean NAS values are shown for subjects above and below SF 1.5 × ULN according to each hepatic iron deposition pattern, including subjects without iron deposition: HC, RES, and mixed HC/RES groups. Significant differences between groups, determined using multivariate ordinal regression modeling after controlling for covariates BMI, age, sex, type 2 diabetes, and serum ALT level, are indicated by arrows. Standard deviations are indicated by the error bars.

independently associated with increased risk of advanced fibrosis in NAFLD patients. Our results indicate that use of a SF value >1.5 × ULN can add diagnostic value to the evaluation of patients with NAFLD. Unlike other case-control or epidemiologic survey studies making comparisons to nondisease control subjects, we did not find any differences in the presence of diabetes or metabolic syndrome between healthy and elevated SF groups, presumably because of the large proportion of NASH CRN subjects with these conditions.[5-8] BMI and SF were inversely related in our study. We speculate that this may be related to the production of hepcidin, the iron-regulatory hormone, from adipose tissue.[51] Hepcidin reduces iron absorption and recycling by binding to and internalizing the iron export protein ferroportin in enterocytes, macrophages, and hepatocytes.[52] Therefore, patients with higher BMI, and thus more body fat, would have more circulating hepcidin as a result of secretion from an expanded fat mass. This, in turn, would result in lower body iron stores and an associated decreased SF level.

A number of previous studies have examined the frequent presence of hyperferritinemia with or without increased hepatic iron storage in patients with NAFLD.[31-43] It was suggested that increased hepatic iron stores, possibly via mutations in *HFE*, the hemochromatosis gene, may lead to NASH and advanced fibrosis in NAFLD via increased oxidative stress.[53] In addition, recent murine studies suggest that hepatic iron loading or *Hfe* mutations, in combination with a high-fat diet, result in up-regulation of genes involved in cholesterol or lipid biosynthesis, providing another mechanism relating iron with NAFLD.[54,55] Another early study suggested that a large proportion of subjects referred for evaluation of hyperferritinemia had NASH, increased hepatic iron stores, features of metabolic syndrome despite normal TS, and absence of *HFE* mutations.[34] The emerging understanding of the intersection between abnormal iron metabolism and NAFLD has proven more complex, demonstrating that many patients with NAFLD had increased SF levels in the absence of increased hepatic iron stores or increased prevalence of *HFE* mutations.[31,32,34,42,49,56] In the current study, hyperferritinemia was associated with a higher HC and RES iron stain grade; however, there was no association with *HFE* genotype and SF >1.5 × ULN was associated with a higher NAS, even among subjects negative for iron staining. In comparison, Bugianesi et al. found SF to be a marker of increased histologic severity, but not of increased hepatic iron concentration.[32] Most NAFLD patients in their study were lean males, and information about features of metabolic syndrome was not reported; finally, SF elevations appeared to be modest, compared to the current series. The U.S. population enrolled in the NASH CRN studies, by contrast, is predominantly overweight or obese and has features of metabolic syndrome.[44-46] These may explain the differences among our studies. It is noteworthy that the clinical and demographic features of a more recent cohort study from Italy examining risk factors for NASH and advanced fibrosis appear much more similar to the NASH CRN.[35] The investigators describe a similar relationship between multiples of SF level >ULN, NASH diagnosis, and severity of hepatic fibrosis. Although the relationship between SF level and advanced histologic features was not examined separately among patients with and without stainable iron, the similarities between these two studies suggest that our results may be applicable to other populations with NAFLD. In fact, recent studies in smaller cohorts from Japan and the UK have found similar results.[38,42]

Although several previous reports have attempted to create discriminant models to identify the presence or absence of severe disease, these are not widely used because of the need for specialized data (i.e., type IV collagen[40]) or the need for use of a complex model that requires multiple variables.[33,57] Although SF has been identified as an independent marker for advanced fibrosis in regression models, previous studies have not examined whether threshold SF levels may provide value to clinicians. The current study provides evidence that SF may be a simple, useful adjunctive marker to clinicians in evaluation of patients with

NAFLD. Another novel aspect of our study is the observation that elevated SF may identify patients with NAFLD who have more severe disease independent of the presence of iron overload. Furthermore, among patients with stainable hepatic iron, elevated SF was a marker for more severe disease.

It is unclear whether hyperferritinemia in NAFLD is simply a consequence of disease severity or actively contributes to disease progression in NASH. Indeed, a number of signals thought to mediate NASH pathogenesis are also known to up-regulate ferritin, including proinflammatory cytokines TNF-α,[17-19,23] interleukin (IL)-1β,[18,20,23] and nuclear factor kappa light-chain enhancer of activated B cells (NFκB)[21,22] and oxidative stress.[25-29] However, recent research suggests that there are a number of potential mechanisms whereby ferritin could actively contribute to NASH pathogenesis. For example, ferritin has been shown to inhibit the secretion of apolipoprotein B *in vitro*,[58] an observation that may, in part, explain the association between ferritin level, degree of steatosis, and increased NAS in this study through decreased very-low-density lipoprotein secretion and increased lipotoxicity.[59] Hepatocyte-secreted ferritin has been shown to promote Fas-mediated apoptosis.[60] Interestingly, a recent study has demonstrated that ferritin can act as a proinflammatory cytokine in activated hepatic stellate cells, in an iron-independent fashion, through induction of a signaling cascade involving phosphoinositide 3-kinase, protein kinase Cζ, mitogen-activated protein extracellular signal-related kinase 1/2, and mitogen-activated protein kinase.[61] This, in turn, resulted in phosphorylation of IκB kinase α/β, activation of the p50/p65 NFκB heterodimer, and the subsequent increased expression of NFκB-responsive genes known to be involved in hepatic fibrogenesis, including IL-1β, inducible nitric oxide synthase, regulated on activation normal T cell expressed and secreted, and intracellular adhesion molecule 1.[61] In summary, ferritin may be intimately involved in many important processes related to NASH pathogenesis, including inflammation, apoptosis, oxidative stress, lipid transport, and fibrogenesis.

SF has also recently been shown to be a predictor of mortality in patients with end-stage liver disease (ESLD), both before[62,63] and after liver transplant.[63] Using SF cut-off values similar to the present study, these large, multicenter studies have shown that hyperferritinemia with ESLD from all etiologies is associated with increased mortality after adjusting for a variety of potential confounding variables.

We recognize limitations to the current study. It can be argued that the threshold SF levels were arbitrarily selected. This is an inherent limitation with any study using "cut-off" levels. However, we set these threshold levels *a priori* and not from a post-hoc analysis. Though we have adjusted the ULN in this study to be sex specific, we recognize that, in practice, the "normal" reference range does vary somewhat between clinical labs and may also be influenced by age—facts that should be kept in mind when interpreting absolute SF values in NAFLD patients.

In summary, we have shown that elevated SF $>1.5 \times$ ULN is associated with hepatic iron deposition, a diagnosis of NASH, and worsened histologic activity and is an independent predictor of advanced hepatic fibrosis among patients with NAFLD. We suggest that SF, an inexpensive, convenient clinical test, should be included in the laboratory evaluation of NAFLD patients.

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