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## Serum Hepcidin Levels Are Associated With Obesity but Not Liver Disease

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### Abstract

**Objective**—Hepcidin is regulated by anemia and inflammation. It is primarily expressed in the liver but studies have reported its expression in adipose tissue. We investigated the relationship between BMI and serum hepcidin and also examined the relationship between liver histology and serum hepcidin in the morbidly obese.

**Design and Methods**—Serum and liver tissue from patients undergoing bariatric surgery (bariatric cohort, n=105) and serum from healthy blood donors (n=60) were used to conduct this study. Serum hepcidin was measured using sandwich ELISA, highly specific for hepcidin-25. Serum ferritin, IL-6, IL-1 $\beta$  and liver function biochemistries were also measured.

**Results**—After controlling for covariates, BMI  $\geq 35$  kg/m<sup>2</sup> was significantly associated with higher serum hepcidin level compared to individuals with lower BMI groups ( $17.7 \pm 11.5$  vs.  $3.3 \pm 4.7$  ng/ml, P=0.002). Presence of NAFLD was not associated with higher serum levels of hepcidin (multivariate P=0.37) There was no association between serum hepcidin levels and liver histology (presence of steatohepatitis, advanced fibrosis, or NAFLD activity score) in the bariatric cohort.

**Conclusions**—Obesity, but not the presence of NAFLD was associated with serum hepcidin levels. There was no association between serum hepcidin and liver histology in the morbidly obese undergoing bariatric surgery.

### Keywords

Serum hepcidin; obesity; ferritin; non-alcoholic fatty liver disease

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**Conflicts of Interest Disclosures:** The authors have no competing interests.

## Introduction

Hepcidin, a 25 amino acid peptide present in human serum and urine, acts as a key regulator of iron metabolism by binding to the iron transporter ferroportin resulting in internalization and lysosomal degradation (1, 2). Several human studies have elucidated the role of hepcidin in hemochromatosis, iron overload and anemia of chronic disease (3-5). In general, hepcidin expression is upregulated by iron stores, inflammation and endoplasmic stress, and is down-regulated by anemia, hypoxia and oxidative stress (6, 7). More recently, hepcidin is increasingly being recognized as a biomarker for systemic inflammatory state due to upregulation by inflammatory cytokines (2, 8). Hepcidin is primarily expressed in the liver but some studies have reported its expression in both subcutaneous and visceral adipose tissue; albeit at much lower levels (9). There was a close correlation between the hepcidin gene expression in subcutaneous adipose tissue and BMI raising the possibility that adipose tissue could contribute significantly to the overall hepcidin pool in morbidly obese patients (9). Of significant interest was the finding of significant positive associations between markers of inflammation such interleukin-6 (IL-6) and C-reactive protein and adipose tissue hepcidin gene expression levels suggesting that extra-hepatic hepcidin gene expression appears to be more sensitive to inflammation (9). These studies suggest that hepcidin may be a proinflammatory adipokine raising the possibility for its use as a noninvasive biomarker for chronic inflammatory diseases.

The role of hepcidin in non-alcoholic liver disease and its utility as a biomarker for non-alcoholic steatohepatitis (NASH) or non-alcoholic fatty liver disease (NAFLD) histological severity has generated much interest due to lack of any established biomarker. Since NASH is associated with both oxidative stress and proinflammatory cytokines; there has been a great interest to explore the biomarker potential of hepcidin as non-invasive marker for the presence of NASH (10-12). These studies showed variable increase in hepcidin levels in patients with NAFLD and variable correlation with hepatic inflammation and histological severity (10-12). For example, Uysal et al. did not observe any significant difference in the serum hepcidin levels in patients with NASH compared to age matched controls (BMI was similar in NASH and control groups) (11). In contrast, Senates et al. reported significantly higher serum levels of hepcidin in NAFLD patients compared to age and gender matched controls (BMI significantly higher in NAFLD group) (10). Additional analyses in this study did not reveal any relationship between hepcidin levels and histological severity of NAFLD. (10) Furthermore, two pediatric studies showed obese NAFLD children had significantly higher serum hepcidin levels compared to lean children (BMI significantly higher in NAFLD group) (13, 14). In summary, the reported higher levels of hepcidin in NAFLD group have only been reported in the context of a significantly higher BMI further supporting the notion that in patients with NAFLD, serum hepcidin levels may be more representative of adipose tissue mass than severity of liver histology.

Because of the small size of the hepcidin molecule (25 amino acids) and compact structure with 4 disulfide bonds, robust immunoassay has been a challenge. A recent study that examined the relationship between serum hepcidin level and parenchymal iron in a large cohort of NAFLD patients (n=786) used a ELISA based assay (Intrinsic Life Sciences, San Diego, CA) with lower limit of detection of 5 nanograms/mL.(15) Up to 2% of the subjects

in this cohort had undetectable serum hepcidin levels (15). Konrad et al. have previously reported that the use of 2 monoclonal antibodies in a sandwich ELISA format provides a robust and convenient method for measuring concentrations of the active form of hepcidin having a limit of quantification that is several fold lower at 0.01 µg/L (0.001 nanograms/mL) (16, 17). The use of this assay with a very low lower limit of quantification is particularly important to measure the levels of serum hepcidin in healthy patients where the levels have previously reported to be low (16, 17). We conducted the following study with two objectives: (a) to examine the relationship between BMI and serum hepcidin in a cohort of individuals with varying degrees of BMI and (b) examine the relationship between liver histology and serum hepcidin in a cohort of patients at high risk for NAFLD.

## Methods

This study was approved by the Institutional Review Board at Indiana University School of Medicine. Consecutive patients undergoing bariatric surgery meeting predefined eligibility criteria (negative viral and autoimmune serology with no significant alcohol consumption) underwent an intraoperative liver biopsy. Anthropometric measures such as weight, height, waist circumference and health information along with a fasting blood sample were obtained before taking them to the operating room. Liver biopsy slides were prepared with hematoxylin and eosin and Masson's trichrome stains. Histological diagnosis of NASH was assessed by an experienced hepatopathologist (R.S) who assessed for the presence of hepatic steatosis and steatohepatitis (borderline or definite). NAFLD activity score (NAS) and extent of fibrosis was scored using fibrosis scores (0, 1a, 1b, 1c, 2, 3 and 4), similar to previously published criteria by the NASH CRN.(18) Blood samples from healthy blood donors were obtained from a local blood bank. These blood donors had (a) normal liver biochemistries, (b) negative serologies for viral hepatitis B and C, (c) no significant co-morbidities, and (d) reported no alcohol consumption. Blood donors and bariatric patients with normal liver histology were deemed not to have liver disease. Serum hepcidin was measured as previously described using a sandwich ELISA format that is highly specific for hepcidin-25 with limit of quantification of 0.01 µg/L (10 pg/mL) (16). Serum ferritin was measured using ELISA kits (ALPCO Diagnostic, Salem, NH 03079, USA) according to manufacturer's instructions. Liver function biochemistries (AST, ALT, Alkaline phosphatase, and total bilirubin) were performed at Indiana University Health Pathology Laboratory.

## Statistical analysis

Descriptive statistics, including means, standard deviations (SD), standard error of mean (SE) and percentages, were used to characterize the study patients and serum biochemical parameters. Comparisons between bariatric cohort and control group were made by the use of Student t-test for the continuous and  $\chi^2$  test for the categorical variables. Pearson's correlation coefficients were used to determine the degree of concordance between any 2 variables. Correlations were considered significant at the  $< 0.05$  level (2 tailed). One-way analysis of variance (one-way ANOVA) with Tukey post-hoc test was used to compare means between the groups (using the F distribution). Univariate linear regression analysis was performed to identify potential predictors of serum hepcidin levels and the variables

with a P value  $> 0.1$  in the univariate analysis were then entered into multivariate analysis to identify independent predictors. Quality control procedures, database management, and statistical analyses were performed using statistical package IBM SPSS Statistics Version 19 (IBM Corporation, Route 100, Somers, NY 10589).

## Results

One hundred and five eligible morbidly obese individuals undergoing bariatric surgery and 60 consecutive eligible blood donors were included. The mean ( $\pm$  S.D) age of the bariatric cohort was  $46 \pm 10$  years with a BMI of  $46 \pm 7$  kg/m<sup>2</sup>; 88% were women; 91% were Caucasian. The mean ( $\pm$  S.D) age of the blood donors was  $51 \pm 13$  years with a BMI of  $30 \pm 6$  kg/m<sup>2</sup>; 23% were women and 93% were Caucasian. Compared to the bariatric cohort, blood donors had significantly lower mean BMI ( $30 \pm 6$  vs.  $46 \pm 7$  kg/m<sup>2</sup>,  $P < 0.001$ ) and lower serum ALT ( $18 \pm 7$  vs.  $29 \pm 21$  U/L,  $P < 0.001$ ), serum hepcidin ( $2.9 \pm 3.5$  vs.  $19.0 \pm 11.0$  ng/ml,  $P < 0.001$ ) and serum ferritin ( $11.8 \pm 13.6$  vs.  $50.4 \pm 48.8$  ng/mL,  $P < 0.001$ ) (Table 1).

### Relationship between serum hepcidin level and BMI

The study participants were stratified based on International Classification of adult overweight and obesity according to BMI cut-off points: normal range  $< 25$  ( $n=14$ ); overweight:  $25-29.9$  ( $n=22$ ); obesity class I:  $30-34.9$  ( $n=16$ ); obesity class II:  $35-39.9$  ( $n=24$ ); and obesity class III  $40$  ( $n=89$ ) as per the WHO 1998 classification.(19) There was a statistically significant difference in the serum levels of hepcidin across different groups of BMI ( $P < 0.001$ ) (Figure 1). Post-hoc multiple comparison test showed a significantly higher serum hepcidin level in Obese class II and III (BMI  $\geq 35$ ) compared to individuals who are overweight and those with normal BMI ( $17.7 \pm 11.5$  vs.  $3.3 \pm 4.7$  ng/mL,  $P=0.002$ ). There was a significant relationship between serum levels of hepcidin and BMI as a continuous variable ( $r=0.51$ ,  $P < 0.001$ ) (Figure 2).

Univariable linear regression analysis revealed that BMI ( $P < 0.0001$ ), ferritin ( $P < 0.0001$ ), total bilirubin ( $P < 0.0001$ ), ALT ( $P=0.087$ ), presence of NAFLD ( $P < 0.001$ ) and female gender ( $P < 0.001$ ) were positively associated with serum hepcidin levels (Table 2). Multivariate analysis revealed BMI ( $\beta = 0.27$ ,  $P < 0.001$ ) and serum ferritin levels ( $\beta = 0.48$ ,  $P < 0.001$ ) were independently associated with serum hepcidin level (Table2).

### Relationship between serum hepcidin and liver histology

Liver biopsy was available in 99 subjects and their histological examination revealed 42 subjects with no significant histological abnormality, 13 subjects with hepatic steatosis, and 44 subjects with steatohepatitis (definite NASH=37; borderline NASH=7) with varying degrees of fibrosis (Table 3). The fibrosis score was 0 in 20 subjects, 1a/1b/1c in 15 subjects, 2 in 6 subjects, 3 in 2 subjects and 4 in 1 subject. Subjects in the NAFLD subgroup were categorized based on the histological severity into hepatic steatosis ( $n=13$ ), NASH with no evidence of fibrosis ( $n=20$ ) and NASH with any fibrosis (F1-F4) ( $n=24$ ). There was no difference among individuals with normal liver histology, steatosis, and NASH in terms of various demographics and clinical characteristics such as age, gender, race, BMI, waist

circumference and prevalence of type2 diabetes (Table 3). Individuals with NASH had significantly higher aspartate aminotransferase (AST) (P=0.017), alanine aminotransferase (ALT) (P=0.002) and gamma-glutamyl transpeptidase (GGT) (P=0.005). However, total bilirubin and alkaline phosphatase (Alk P) were not statistically significantly different between the groups (Table 3). Serum hepcidin levels were not significantly different among these bariatric subgroups. Serum ferritin (P=0.013) was statistically significantly higher in NASH with fibrosis group (Figure 3A). However, levels of serum hepcidin in these subgroups were not significantly different (Figure 3B).

## Discussion

The need to investigate the mechanistic link between obesity and hepcidin has garnered much attention since Bekri et al. reported higher hepcidin mRNA expression in adipose tissue without a significant change in liver hepcidin mRNA of obese premenopausal women (9). This observation led them to hypothesize that the sizeable subcutaneous and adipose tissue mass in obesity may be responsible for alterations in iron status observed with obesity (9). Subsequently, investigators have reported that overweight children had higher circulating serum hepcidin and poorer iron status when compared to normal weight children (13, 20). Furthermore, weight loss interventions were associated with a decrease in serum hepcidin level and concomitant increase in iron absorption (21). A recent interest has been to evaluate the role of hepcidin as a biomarker for NAFLD severity due to the regulation of hepcidin expression by inflammation and oxidative stress, two critical mechanisms implicated in the pathogenesis of NASH.

In our study, higher BMI was associated with higher levels of serum hepcidin in a fashion that is independent of liver disease. This observation suggests that synthesis of hepcidin by adipose tissue may be higher in obesity. But this speculation can only be confirmed by comparing the adipose tissue hepcidin expression between lean and obese individuals. Prior studies examining the serum hepcidin levels in NAFLD patients (10, 11) have shown inconsistent results, likely due to different BMI between the patient groups. However, two recent studies reported that adipose tissue may actually contribute very little to circulating serum hepcidin levels based on lower levels of adipose compared to liver hepcidin mRNA expression (22, 23). In addition, we observed a wide variability in serum hepcidin levels in individuals with similar BMI in obese individuals (Figure 2) possibly due to complex interaction between visceral and peripheral adipocyte and liver hepcidin production in the context of inflammation, iron deficiency and oxidative stress as the net circulating hepcidin level is regulated by these positive and negative stimuli.

Serum hepcidin levels were also not predictive of histological severity of NAFLD or the diagnosis of NASH. Our findings further support the recent report of failure to find any association between serum hepcidin and histological staging or pathological characteristics of NAFLD (10). Lack of association between serum hepcidin levels and severity of NAFLD is likely related to confounding effect of adipocyte tissue's contribution to total serum hepcidin. Another finding in the current study was significantly elevated serum ferritin levels in the patients with NASH with fibrosis compared to hepatic steatosis or NASH without fibrosis. Although not novel, this finding is consistent with a recent study that

showed a significant association between elevated serum ferritin and a diagnosis of NASH, higher histologic activity, and advanced hepatic fibrosis among patients with NAFLD (15). This relationship was evident even in patients without iron overload suggesting that ferritin is more likely representative of the chronic inflammatory state associated with progressive NAFLD.

Samples of blood for the control group are from self-reported healthy individuals and were only screened for infectious hepatitis and liver biochemistries. Although majority of the subjects in the control group had BMI < 30 kg/m<sup>2</sup>, some had extreme obesity with BMI >35 kg/m<sup>2</sup> (17%) and NAFLD cannot convincingly be excluded without some form of imaging in this subgroup. It is very unlikely that this limitation would challenge the validity of our findings. Other issues such as surgical hepatitis and postoperative increase in the level of hepcidin (i.e., a hepcidin storm) were avoided in the current study by collecting venous blood sampling in the preoperative area and by obtaining liver biopsy prior to performing bariatric surgery (24). The recently reported association of significant increase in serum hepcidin to ferritin ratio in cirrhotics could not be validated as only one patient had cirrhosis in our study (25).

In summary, our study found a significant association between obesity and serum hepcidin that is independent of liver disease. It did not find a significant relationship between serum hepcidin and liver histology among the morbidly obese undergoing bariatric surgery. Further studies need to be performed to determine the factors that contribute to the total hepcidin pool and the effect of liver disease severity.

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## Abbreviations

<b>BMI</b>	Body Mass Index
<b>ELISA</b>	Enzyme-linked immunosorbent assay
<b>NASH CRN</b>	Non-alcoholic steatohepatitis Clinical Research Network
<b>NAFLD</b>	Non-alcoholic fatty liver disease
<b>AST</b>	Aspartate aminotransferase
<b>ALT</b>	Alanine aminotransferase

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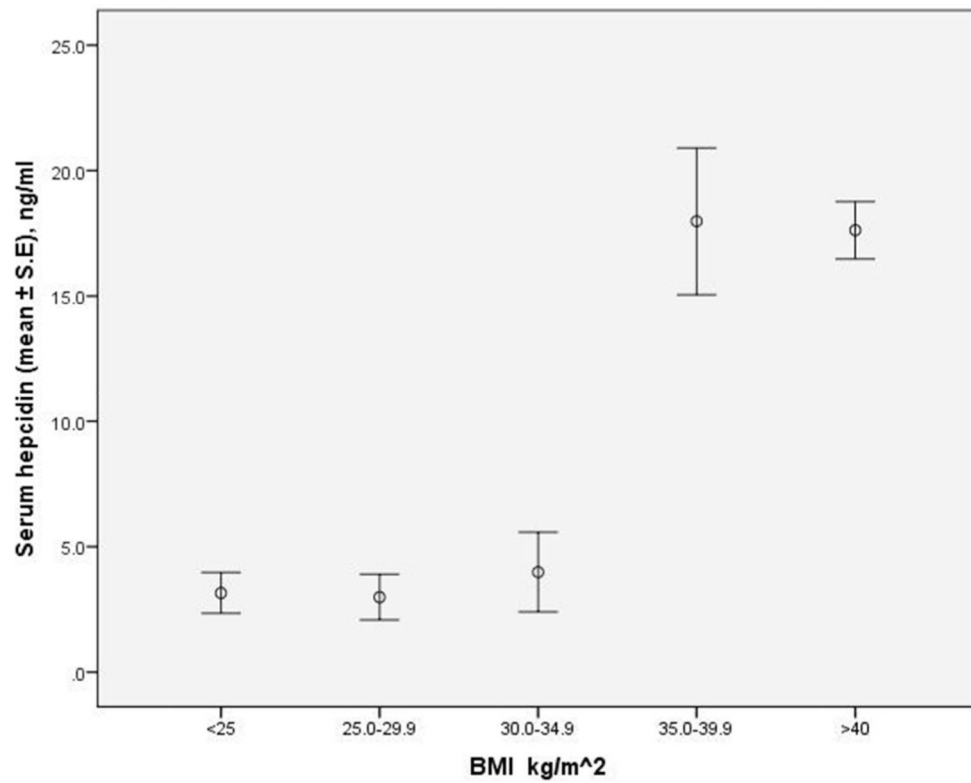


**What is already known about this subject?**

1. Hepcidin is primarily expressed in the liver and is regulated by anemia and inflammation.
2. Some studies have reported hepcidin expression in both subcutaneous and visceral adipose tissue; albeit at much lower levels.
3. Studies examining the relationship between serum hepcidin levels and severity of non-alcoholic fatty liver disease reported conflicting data.

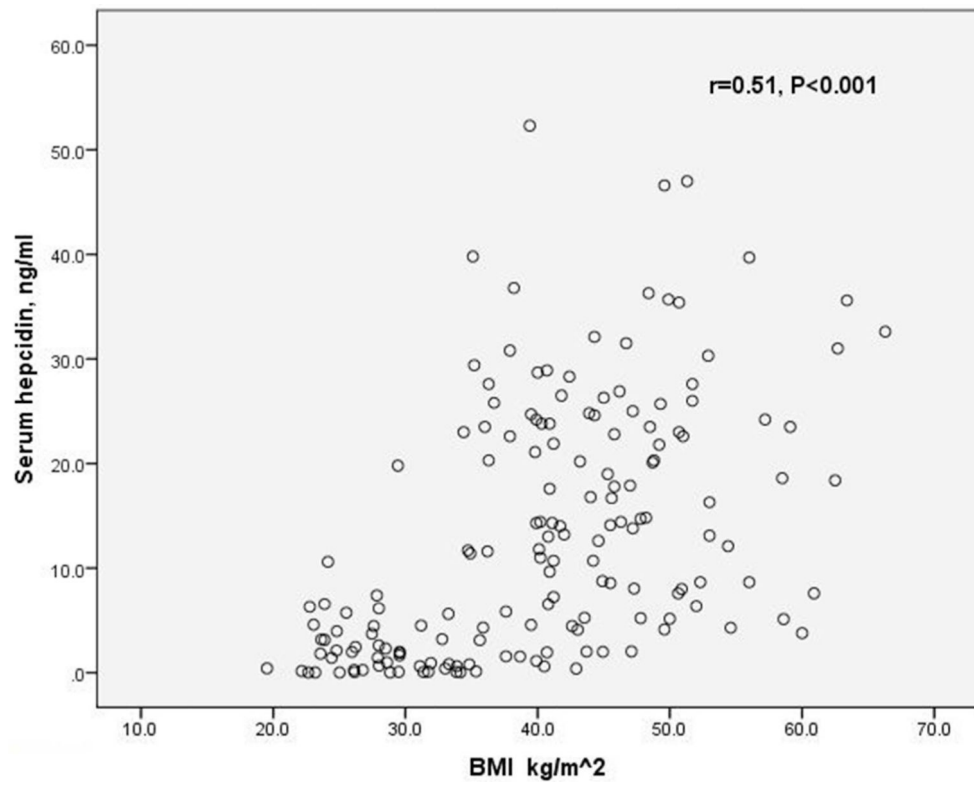
**What does this study add?**

1. There is positive correlation between body mass index and serum hepcidin levels.
2. There was no association between serum hepcidin and severity of NAFLD liver histology.
3. Obesity, but not the presence of NAFLD was associated with serum hepcidin levels.

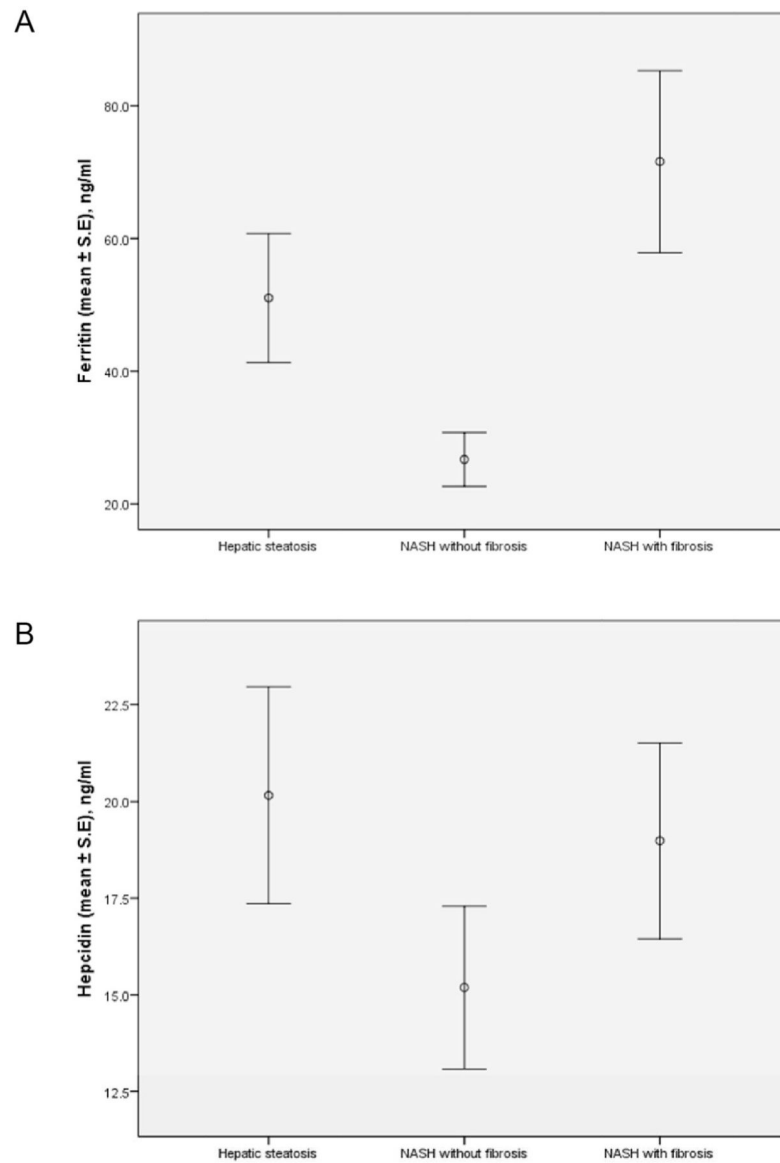


**Figure1.**

The study participants were stratified based on International Classification of adult overweight and obesity according to BMI cut-off points: Normal range <25 (n=14); Overweight: 25-29.9 (n=22); Obese class I: 30-34.9 (n=16); Obese class II: 35-39.9 (n=24); and Obese class III 40 (n=89) as per the WHO 1998 classification. ANOVA showed a significant relationship between BMI and serum hepcidin levels ( $P<0.001$ ). Post-hoc multiple comparison test showed significantly higher serum hepcidin levels in the obesity class II and III (BMI  $\geq 35$ ) compared to individuals with normal BMI and obesity class I ( $P<0.001$ ). There was no difference between obesity classes II and III ( $P=1.0$ ).



**Figure2.** Significant correlation between serum hepcidin level and BMI as a continuous variable ( $r=0.51$ ,  $P<0.001$ ).



**Figure 3.** Individuals with NAFLD in bariatric cohort (n=57) were categorized based on the histological severity into hepatic steatosis (n=13), NASH with no evidence of fibrosis (n=20) and NASH with any fibrosis (F1-F4) (n=24). Serum ferritin ( $P=0.013$ ) was statistically significantly higher in NASH with fibrosis group (Figure 3 A) compared to the other subgroups. However, levels of serum hepcidin in these subgroups were not significantly different (Figure 3B).

**Table 1**  
**Select demographic and serum biochemical parameters in the bariatric and blood bank control cohorts. Values expressed are mean  $\pm$  standard deviation unless otherwise mentioned**

	<b>Bariatric cohort (n=105)</b>	<b>Healthy control (n=60)</b>	<b>P</b>
<u>Demographics</u>			
Age (years)	46 $\pm$ 11	51 $\pm$ 13	0.014
Female (%)	88	23	<0.001
Caucasian (%)	91	97	0.33
BMI (kg/m <sup>2</sup> )	46 $\pm$ 7	30 $\pm$ 6	<0.001
<u>Serum biochemical parameters</u>			
AST (U/L)	26 $\pm$ 18	25 $\pm$ 6	0.49
ALT (U/L)	29 $\pm$ 21	18 $\pm$ 7	<0.001
Alkaline phosphatase (U/L)	71 $\pm$ 22	62 $\pm$ 13	0.002
Total Bilirubin (mg/dL)	0.7 $\pm$ 0.2	0.5 $\pm$ 0.2	<0.001
Serum hepcidin (ng/ml)	19.1 $\pm$ 11.0	2.9 $\pm$ 3.5	<0.001
Serum ferritin (ng/ml)	50.4 $\pm$ 48.8	11.8 $\pm$ 13.6	<0.001

BMI = Body Mass Index; AST = aspartate aminotransferase; ALT = alanine aminotransferase.

**Table2**  
**Variables associated with serum hepcidin levels in the bariatric cohort: Summary of univariable and multivariable analysis**

	Univariable analysis		Multivariable analysis	
	Parameter estimate	P	Parameter estimate	P
BMI (kg/m <sup>2</sup> )	0.51	<0.001	0.27	<0.001
Total Bilirubin (mg/dL)	0.36	<0.001	0.08	0.19
ALT (U/L)	0.13	0.09	-0.02	0.71
Ferritin (ng/ml)	0.63	<0.001	0.48	<0.001
Presence of NAFLD	0.31	<0.001	0.06	0.37

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**Table3**  
**Select demographic, biochemical, hematological parameters, ferritin and hepcidin in the bariatric cohort. Values expressed are mean  $\pm$  standard deviation unless otherwise mentioned**

	Bariatric cohort subgroups			P
	Not NAFLD (n=42)	Steatosis (n=13)	NASH $\pm$ fibrosis (n=44)	
<u>Demographics</u>				
Age (years)	45 $\pm$ 11	46 $\pm$ 13	47 $\pm$ 9	0.86
Female gender (%)	91	85	89	0.82
Caucasian race (%)	88	85	98	0.08
BMI (kg/m <sup>2</sup> )	45 $\pm$ 7	45 $\pm$ 8	47 $\pm$ 7	0.47
Waist circumference (cms)	129 $\pm$ 12	132 $\pm$ 16	135 $\pm$ 14	0.09
Diabetes type 2 (%)	31	46	36	0.59
Hypertension (%)	67	69	73	0.83
Dyslipidemia (%)	33	62	59	0.04
<u>Biochemical parameters</u>				
AST (U/L)	22 $\pm$ 5	23 $\pm$ 6	32 $\pm$ 26	0.02
ALT (U/L)	22 $\pm$ 6	26 $\pm$ 11	37 $\pm$ 29	0.002
Alkaline phosphatase (U/L)	72 $\pm$ 20	64 $\pm$ 13	72 $\pm$ 27	0.53
GGT (U/L)	20 $\pm$ 7	25 $\pm$ 15	40 $\pm$ 40	0.005
Total Bilirubin (mg/dL)	0.8 $\pm$ 0.2	0.7 $\pm$ 0.3	0.7 $\pm$ 0.2	0.37
Creatinine (mg/dL)	0.8 $\pm$ 0.2	1.0 $\pm$ 0.2	0.9 $\pm$ 0.2	0.06
<u>Hematologic parameters</u>				
Hemoglobin (g/dL)	13.6 $\pm$ 1.0	13.6 $\pm$ 1.1	13.7 $\pm$ 3.5	0.99
Hematocrit (%)	39.9 $\pm$ 2.8	39.7 $\pm$ 2.5	39.9 $\pm$ 3.2	0.98
Iron saturation (%)	18.4 $\pm$ 6.9	18.5 $\pm$ 6.7	15.5 $\pm$ 7.6	0.14
Platelet count ( k/mm <sup>3</sup> )	266 $\pm$ 64	288 $\pm$ 47	315 $\pm$ 98	0.02
Serum hepcidin (ng/ml)	19.4 $\pm$ 10.3	20.2 $\pm$ 10.1	17.3 $\pm$ 11.2	0.56
<u>Markers of inflammation</u>				
IL6 (pg/ml)	4.33 $\pm$ 2.82	4.85 $\pm$ 3.50	6.09 $\pm$ 4.34	0.08
IL1 $\beta$ (pg/ml)	0.46 $\pm$ 1.07	0.52 $\pm$ 0.78	0.94 $\pm$ 1.28	0.13
Serum ferritin (ng/ml)	49.5 $\pm$ 48.9	51.0 $\pm$ 35.1	51.2 $\pm$ 55.5	0.99