Role of Serum and Urinary Hepcidin in Young Females of Reproductive Age in North India

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Abstract

Introduction   Iron deficiency is one of the most common nutritional disorders in the world affecting young females of the reproductive age group. Indeed, an ideal screening test should be capable of identifying iron deficiency long before developing anemia. Henceforth, the present study was aimed to determine utility of hepcidin in iron deficiency and to see its correlation with different iron indices.

Methods   This cross-sectional study was conducted in the Department of Biochemistry, SGT Medical College Hospital and Research Institute, Budhera, Gurugram, Haryana, India. It included 200 nonpregnant female students aged between 18 and 25 years. Estimation of hepcidin was by enzyme-linked immunosorbent assay. Quantitative estimation of serum iron, total iron-binding capacity (TIBC), and transferrin saturation was done via semi-autoanalyzer. Statistical analysis was done using SPSS v22.

Results   The reference range of urinary hepcidin established in this study was 110 to 969 ng/mg creatinine (mean ± standard deviation 328.3 ± 195.07 ng/mg creatinine). Serum hepcidin and urinary hepcidin had a significant correlation with iron indices. Area under the curve of urinary hepcidin was obtained with best combination of diagnostic sensitivity (82.6%) and specificity (83.1%) at a cutoff value of > 15.7 ng/mL and ≤ 199 ng/mg, respectively.

Conclusion   Since ferritin, TIBC, transferrin saturation, and hepcidin each represent different aspects of iron metabolism, incorporating hepcidin in the present diagnostics and combined evaluation of these indices may accord enhanced clinical information. Hepcidin would help to stratify the vulnerable young healthy female population in early stages of iron deficiency and guide proper interventions to reduce morbidity.

Keywords
► iron deficiency
► hepcidin
► transferrin
► young females

Introduction

Anemia is one of the most common nutritional disorder affecting 1.62 billion people worldwide. It is a public health problem affecting globally with major impact on health as well as socioeconomic development. Majority of the cases are seen in developing countries and among these, about half of the cases are due to iron deficiency (ID). It is estimated that around 52% of nonpregnant women of reproductive age are anemic in India.[1,2] Maintenance of body iron status is an integral part of healthcare in young females of reproductive age group. Therefore, early detection of iron deficiency anemia (IDA) can lead to early intervention.

However, commonly used tests for diagnosis of iron status have their limitations. Ferritin is an indicator of iron stores, but it is an acute phase reactant, levels of which increase in inflammation.[3] Similarly, soluble transferrin receptor (sTfR) levels reflect tissue ID, but are affected by erythropoietic activity.[4] In addition, transferrin saturation (TSAT) levels may be influenced by inflammation and undergo diurnal variation.[5]

Hepcidin-25, a 25-amino acid peptide hormone produced in the liver, is a central hub of systemic iron metabolism.[6–9] Hepcidin downregulates iron absorption in duodenum and iron release from macrophage by modulating cellular iron export via ferroportin.[10] Therefore, dysregulation of hepcidin production may lead to a variety of iron disorders.[11] Hepcidin levels are reduced in patients with ID. Therefore, measurement of hepcidin levels may act as a determinant of iron bioavailability and an accurate indicator of physiological ID. Further, urinary hepcidin reflects an indirect assay of the circulating hormone level and a noninvasive method. Currently, hepcidin testing is not incorporated in routine iron profile markers in diagnostic laboratories. Blood and urinary hepcidin may provide as a screening tool for iron disorders. The present study was aimed to determine utility of hepcidin as an indicator of ID in nonpregnant nonanemic females and explore its association with other routine iron profile markers.

Materials and Methods

This cross-sectional study was conducted in the Department of Biochemistry, SGT Medical College Hospital and Research Institute, Budhera, Gurugram, Haryana. Two-hundred nonpregnant female students (age 18–25 years), who voluntarily participated in the study and had hemoglobin > 12 g% and normal red blood cell (RBC) indices, were included in the study. However, female students on iron therapy, oral contraceptive pills, with chronic liver disease, any systemic illness, and acute or chronic infection were excluded from the study.

Sample Collection and Processing

Under strict aseptic precautions, 3 mL of venous blood was collected in ethylenediaminetetraacetic acid vacutainers that was used for the estimation of white blood cell, RBC, hemoglobin [Hb], hematocrit, mean corpuscular volume, mean corpuscular hemoglobin concentration [MCHC], platelet count, MCH via the SYSMEX KX 21 autoanalyzer and 5 mL was drawn in plain vacutainers that was centrifuged to separate serum. Quantitative estimation of serum iron, total iron-binding capacity (TIBC), transferrin, and sTfR was done by semiauto analyzer (ERBA CHEM 7) using Spinreact, S.A, Spain diagnostic kit. TSAT was calculated using the formula serum iron × 100/TIBC. The serum was stored at –20°C that was used to measure serum hepcidin level. Midstream urine samples were collected in the morning between 8 and 10 AM to avoid diurnal variation. Then samples were centrifuged and were stored in aliquots at −20°C. To account for the variable dilution of urine samples, hepcidin concentration was normalized to urinary creatinine.

Hepcidin estimation was done by enzyme-linked immunosorbent assay (human hepcidin, ELISA Kit, Sincere Biotech Co., Ltd, Beijing, China). Purified human hepcidin antibody was used by the manufacturer to coat the microtiter plate wells to make solid-phase antibody. First, the serum sample was poured in the microtiter wells. Human hepcidin present in the serum sample combined with the coated antibody. Horse radish peroxidase (HRP) labeled antibody was then added to form antibody-antigen-enzyme-antibody complex. After washing, 3,3′,5,5′-tetramethylbenzidine substrate solution was added that changed to blue color. HRP enzyme-catalyzed reaction was terminated by the addition of a sulfuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450 nm. Thereafter, concentration of human hepcidin in the serum samples was determined by comparing the optical density of the samples to the standard curve.

Statistical Analysis

Data was analyzed using SPSS 20.0 version. Parametric data were represented by mean and standard deviation (SD), whereas nonparametric data were represented by median and quartile. Correlation between hepcidin and other parameters of iron status was determined by Spearman Correlation Test. Overall comparisons between serum and urinary hepcidin in each of the three ferritin groups (severe deficiency, moderate deficiency, and normal) were evaluated by Kruskal–Wallis test. Receiver operating curve (ROC) analysis and area under curve (AUC) were done to test the cutoff value of hepcidin in subclinically iron-deficient females. For all tests, a p-value of less than 0.05 was considered significant.

Results

Female students volunteering from SGT University conforming to the inclusion and exclusion criteria were screened for study. Screening was continued till the appropriate sample size for the study was reached. A total of 232 students were screened, of which 32 students were excluded because of low hemoglobin < 12 g/dL. Seven of the excluded students had body mass index (BMI) ≥ 30. Thus, 200 healthy nonpregnant female university students were taken for the study.

Table 1 Hematologic parameters and iron status of the study group based on iron profile parameters of study group

Parameter	Mean ± SD	Normal reference range
Hemoglobin (g/dL)	13.1 ± 0.69	12–15
Hematocrit (%)	40.1 ± 2.11	40–50
RBC count (millions/mm^3)	4.14 ± 0.261	3.5–5.2
MCV (fL)	96.7 ± 1.34	80–100
MCH (pg)	31.6 ± 0.42	27–32
MCHC (g/dL)	32.7 ± 0.03	30–35
TLC (thousand/mm^3)	8028.5 ± 648.34	4,000–10,000
Serum iron (µg/dL)	87.4 ± 23.59	50–150
TIBC (µg/dL)	425.1 ± 16.38	300–360
Serum transferrin (mg/ dL)	297.3 ± 25.62	204–360
TSAT (%)	20.7 ± 6.26	(30–50%)
Serum ferritin (µgm/L)	33.8 ± 21.1	50–200
sTfR (mg/L)	1.88	0.76–2.68
Serum hepcidin (ng/mL)	42.4 ± 29.13	12.14–139.89
Urinary hepcidin (ng/mg creatinine)	328.3 ± 195.07	110–969

Abbreviations: MCHC, mean corpuscular hemoglobin concentration; RBC, red blood cell; TIBC, total iron-binding capacity; TLC, total leucocyte count; TSAT, transferrin saturation; SD, standard deviation; sTfR, soluble transferrin receptor.

Study population comprised of young healthy females with a mean age of 19.4 years and a mean BMI of 25.8. The hemoglobin, hematocrit, and red cell indices of all the subjects were within the normal reference range indicating no evidence of anemia. The total leucocytes count was also within the normal range ruling out any inflammation. Iron status of the study group has been ascertained based on various parameters as depicted in ►**Table 1**. In ►**Table 2**, comparison was made on the basis of different parameters, namely ferritin, TIBC, and TSAT. Serum hepcidin values had a statistically significant positive correlation with hemoglobin, serum iron, serum ferritin, and TSAT% and a statistically significant negative correlation with sTfR, serum transferrin, and TIBC (►**Fig. 1A**, ►**Table 3**).

Urinary hepcidin was in the range of 110 to 969 ng/mg creatinine in this population group with the mean and SD

being 328.3 ± 195.07. Urinary hepcidin values also had a positive correlation with Hb, serum iron, serum ferritin, and TSAT% and a negative correlation with sTfR, serum transferrin, and TIBC; all correlations were highly statistically significant (►**Table 4**, ►**Fig. 2A**). Urinary hepcidin on ROC showed higher discriminating power in evaluating iron status in young healthy women in the age group 18 to 25 years (AUC: 0.857) with best combination of diagnostic sensitivity (82.6%) and specificity (83.1%) at a cutoff value of ≤ 199 ng/mg creatinine (►**Fig. 3**). On evaluation, urinary hepcidin based on cutoffs derived from ROC curve 24.5% (49/200) of the students was found to be iron deficient (►**Table 5**).

Discussion

ID is one of the most common nutritional deficiency and a significant public health problem worldwide largely affecting women and children.[1] Hence, prompt diagnosis and early intervention are essential in women of reproductive age who are the bearers of future generations.

The progression to ID can be divided into three stages. First stage of negative iron balance where demand of iron exceeds the body's ability to absorb iron from the diet, during which serum ferritin levels decrease but the serum iron, TIBC, and red cell protoporphyrin levels remain within normal limits as long as iron stores are present and can replete. Second stage of iron-deficient erythropoiesis when iron stores become depleted and when serum ferritin levels fall to less than 15 µg/L, the serum iron begins to fall, followed by increased TIBC, and TSAT falls to 15 to 20% impaired hemoglobin synthesis. Third stage of IDA, the Hb and hematocrit begin to fall. TSAT is 10 to 15% in this stage. Severe

Table 2 Discrimination between ID and NID based on parameters

Parameter	ID/NID	n = 200	
		n	%
TIBC > 425mg/dL Serum ferritin ≤ 15µg/L	ID	52	38
TIBC ≤ 425 mg/ dL Serum ferritin > 15µg/L	NID	148	62
TSAT ≤ 16% Serum ferritin ≤ 15µg/L	ID	55	31.5
TSAT > 16% Serum ferritin > 15µg/L	NID	145	68.5

Abbreviations: ID, iron deficiency; NID, non-iron deficiency; TIBC, total iron-binding capacity; TSAT, transferrin saturation.
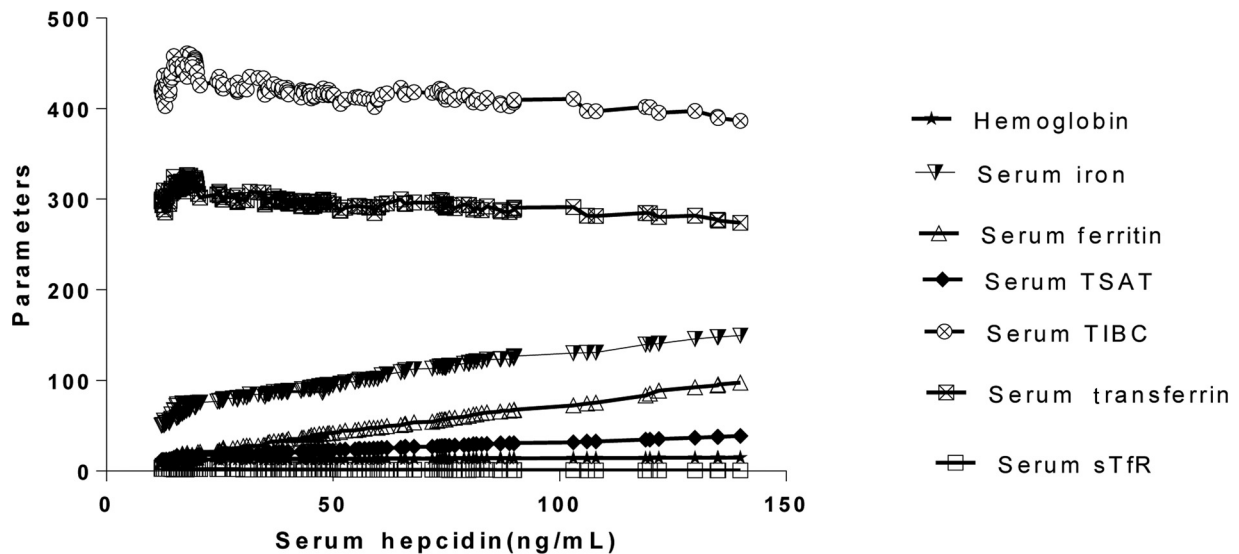
Fig. 1 Correlation between serum hepcidin with hemoglobin, serum iron, serum ferritin and TSAT%, serum transferrin, TIBC, and sTfR. Parameters: Hemoglobin (g/dL); serum iron (µg /dL); serum ferritin (µg/L); serum transferrin saturation (TSAT%); serum total iron-binding capacity (TIBC) (µg/dL); serum soluble transferrin receptor(sTfR) (mg/L).

anemia sets in with microcytosis, hypochromia, target cells, and poikilocytes in blood smear.[12,13]

Since available parameters have their own limitations, their synergism with markers like hepcidin would help to stratify the vulnerable population in early stages of negative iron balance or iron-deficient erythropoiesis and guide proper interventions to reduce morbidity. Hepcidin is the iron-regulatory hormone that plays a cardinal role to maintain homeostasis of extracellular iron concentrations.[14] Hepcidin is produced by hepatocytes that undergoes posttranslational processing by prohormone convertase into its biologically active form hepcidin (25 amino acid peptide).[15] It is rapidly cleared from the circulation. Urinary hepcidin levels correlate well with hepatic hepcidin mRNA. Hepcidin isoforms-20, -22, and -25 are the three isoforms

excreted in urine of which hepcidin-25 and -20 are also found in serum.[16,17] Hepcidin-25 isoform plays predominant role in iron regulation.

Hepcidin inhibits iron entry into the plasma compartment from main sources like the dietary absorption in the duodenum, release of recycled iron from macrophages, and the release of stored iron from hepatocytes by bonding to cellular iron efflux channel, ferroportin, inducing its internalization and lysosomal degradation.[9] N-terminally truncated breakdown products of hepcidin have been detectable in plasma and urine. Urine testing is chosen in preference to serum assays as it is less affected by diurnal variation and the noninvasive nature of sampling.[16,17]

The available methods for IDA diagnosis have their limitations. Ferritin as a marker is raised with age and in chronic

**Table 3** Correlation of serum hepcidin with traditional iron parameters

	Spearman correlation coefficient (r)	p-Value
Serum hepcidin vs. hemoglobin	0.962	< 0.0001
Serum hepcidin vs. serum iron	0.977	< 0.0001
Serum hepcidin vs. serum transferrin	−0.813	< 0.0001
Serum hepcidin vs. TSAT %	0.986	< 0.0001
Serum hepcidin vs. serum ferritin	0.994	< 0.0001
Serum hepcidin vs. sTfR	−0.929	< 0.0001
Serum hepcidin vs. TIBC	−0.763	< 0.0001

Abbreviations: TIBC, total iron-binding capacity; TSAT, transferrin saturation; sTfR, soluble transferrin receptor.

Table 4 Correlation of urinary hepcidin with traditional iron parameters

	Spearman correlation coefficient (r)	p-Value
Urinary hepcidin vs. hemoglobin	0.917	< 0.0001
Urinary hepcidin vs. serum iron	0.861	< 0.0001
Urinary hepcidin vs. serum transferrin	−0.695	< 0.0001
Urinary hepcidin vs. TSAT %	0.864	< 0.0001
Urinary hepcidin vs. serum ferritin	0.970	< 0.0001
Urinary hepcidin vs. sTfR	−0.922	< 0.0001
Urinary hepcidin vs. TIBC	−0.694	< 0.0001

Abbreviations: TIBC, total iron-binding capacity; TSAT, transferrin saturation; sTfR, soluble transferrin receptor.
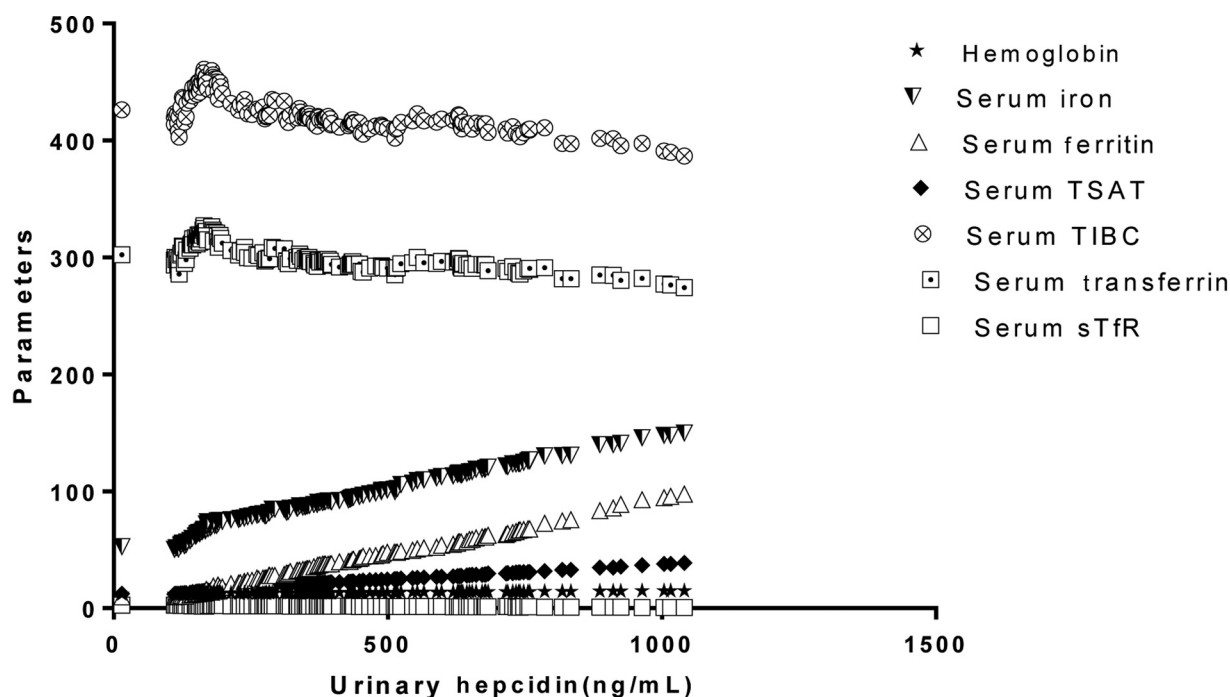
Fig. 2 Correlation between urinary hepcidin with hemoglobin, serum iron, serum ferritin, and transferrin saturation (TSAT%), serum transferrin, total iron-binding capacity (TIBC), and soluble transferrin receptor (sTfR).

infections, malignancies, hepatitis, and hyperthyroidism; so, it is relatively less sensitive in detecting an early ID.[18–20] Moreover, sTfR is affected by erythropoietic activity, whereas TSAT levels may be influenced by inflammation and diurnal variation.[4,5] Bone marrow aspiration for iron studies is an invasive procedure, nonpatient compliant, tedious, and requires aseptic condition; hence, it cannot frequently be used as a method of choice for the detection of iron-deficient anemia.[21,22]

The aim of this study was to determine utility of hepcidin as an indicator of ID in nonpregnant nonanemic females and observe its association with other routine iron profile markers that alter in early stages. The normal reference range of urinary hepcidin in young healthy females and cutoff value was determined.

It was observed in present study that serum iron levels in all the study group students were within the normal reference range. Since serum iron levels show diurnal fluctuation, an isolated serum iron determination is not quite useful to differentiate ID and IDA.[23] Cook and Skikne also reported that serum Iron was suitable only to detect advanced ID as it is subjected to variation in dietary intake, iron therapy, and diurnal variation.[23,24] Our results were comparable to previous studies.[25–27]

Further discrimination between ID and non-iron deficiency (NID) was based on TSAT% and ferritin (TSAT $\leq$ 16% and serum ferritin $\leq$ 15 µg/L were identified as ID; TSAT > 16% and serum ferritin > 15µg/L were identified as NID). Sixty three out of 200 were ID (31.5%), and the rest 137/200 were NID (68.5%). It has been reported that TSAT% measurements are fairly insensitive as the degree of change with variation in iron stores is small relatively to assay variability.[24]

Serum hepcidin was in the range of 12.14 to 139.89 ng/mL in this population group and was associated with good appreciable AUC on ROC curve as reported in our previous study.[28] Reference range of 5.4 to 174.6 ng/mL was observed among healthy premenopausal female in Australia.[29] Similarly, reference range of 17.186 to 91.237 ng/mL for Namibian female blood donor population and 29 to 254 ng/mL was observed by previous studies.[30] Galesloot et al also established normal ranges for hepcidin in the Netherlands with median of 4.1 nM.[21]

Hepcidin is master regulator of iron homeostasis through its effect on ferroportin; therefore, it gives an accurate estimate of iron bioavailability. Incorporating this biomarker into the present diagnostic arena also requires good correlation with other iron parameters that would be beneficial for clinicians in disease management.

Hence, on correlation study it was observed that serum hepcidin showed significant positive correlation with hemoglobin, serum iron, ferritin and TSAT% and negative correlation with sTfR and serum transferrin. Similar correlation was seen in premenopausal female and children.[3,29]

Hepcidin and serum ferritin respond same to the change in iron stores and inflammation and this was reflected in the strong correlation between hepcidin and ferritin. Further, changes in ferritin concentration are much slower but hepcidin responses take place in time scale of few hours. This emphasizes understanding of the pathogenic role of hepcidin in various disorder and application in therapeutic intervention.[3,17,31,32]

The specificity of hepcidin concentration as an index of iron status is reflected by its physiology. ID and erythropoiesis lead to suppressed hepatic hepcidin release,[32] facilitating increased intestinal iron absorption[33] and release from macrophage stores through intact membrane ferroportin on the basolateral aspect of enterocytes[9] and macrophages.
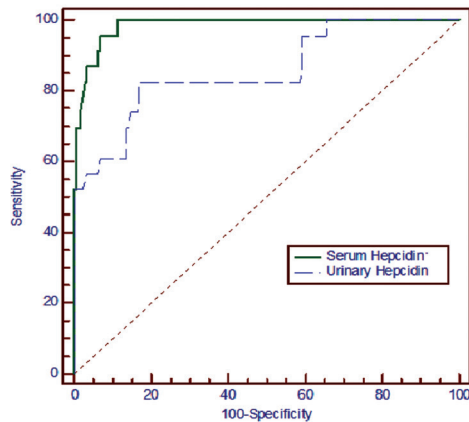
	Serum Hepcidin	Urinary Hepcidin
Optical Cutoff	≤ 15.7	≤ 199
Sensitivity	95.7	82.6
Specificity	93.2	83.1
AUC	0.984	0.857
Std error	0.0075	0.0476
P-value	< 0.001	< 0.001

Fig. 3 Receiver operating curve analysis of serum hepcidin(ng/mL) and urinary hepcidin (ng/mg) in evaluation of iron status ($n = 200$).

Thus, decreased hepcidin is an essential part of the physiological response to an iron deficit. Ferritin (an index of iron stores), sTfR (an index of erythropoietic marrow iron depletion), and hepcidin (a signal that increased iron is required) each display various aspects of iron metabolism. Thus, combined evaluation of these indices may afford complementary clinical information enhancing patient care. A low hepcidin concentration may predict efficient intestinal absorption of supplemental iron,[33] potentially delineating patients who would benefit most from oral iron therapy.

Decreased hepcidin, together with low TSAT and serum ferritin, is observed prior to a detectable decrease in Hb. In conjunction, hepcidin measurements could improve the screening of IDA before development of hematological manifestations that are relatively late sequelae of IDA.

However, diurnal variation and standardization of assays play an important role in estimation of hepcidin. Further utility of serum hepcidin for diagnosing ID should be confirmed in studies with larger sample sizes to assure enhanced diagnoses and clinical management.

Urinary hepcidin may provide an indirect measure of the circulating hormone hepcidin level. Hence, in present study we further determined urinary hepcidin levels that are less affected by diurnal variation and simple noninvasive collection method. However, urinary hepcidin cannot be used in case of kidney disease. In present study, the reference range observed was 110 to 969 ng/mg creatinine with mean ± SD of urinary hepcidin being 368.3 ± 195.07 ng/mg.

Table 5 Discrimination between ID and NID population based on urinary hepcidin

Parameter	ID/ NID	$n = 200$	
		n	Percentage
Urinary hepcidin ≤ 199 ng/mg	ID	49	24.5
Urinary hepcidin > 199 ng/mg	NID	151	75.5

Abbreviations: ID, iron deficiency; NID, non-iron deficiency.

There has been limited studies of urine hepcidin in young females. Urinary hepcidin was observed to be 0.51 to 7.22 intensity/mmol creatinine (mass spectrophotometric estimation) in Bangladeshi women.[34] However, urinary hepcidin levels of 443.92 ± 91.84 ng/ml, with range from 344 to 650 ng/mL[35,36] and 0.62 to 6.65 nmol/mmol creatinine range and cutoff value ≤ 0.94 nmol/mmol creatinine, were reported in children. Median urinary concentration of 502 ng/mg creatinine with the range of 71 to 1,762 was reported by Ganz et al in healthy women volunteers.[17] Therefore, uniform standardization of assays and setting of reference range in different populations may likely help in better prediction of iron status. Similar to serum hepcidin, urinary hepcidin showed significant correlation with iron indices. Present data were in agreement with previous studies.[37,38]

For urinary hepcidin, an AUC of 0.875 was obtained with the best combination of diagnostic sensitivity (82.6%) and specificity (83.1%) at a cutoff value of ≤ 199 ng/mg. About 24.5% of young females were diagnosed to be iron deficient based on urinary hepcidin cutoff value. Reduced urinary hepcidin is a crucial part of the physiological response to an IDA. Decreased hepcidin and serum ferritin along with other routine iron indices are indicators of iron demand. Hence, combined evaluation of Hepcidin along with these iron profile markers may complement each other and enhance diagnosis. Use of urinary hepcidin level may prove as a sensitive, noninvasive investigation that can evaluate iron state in young reproductive females, even in early stages before developing anemia.

One of our limitations in this study was the small number of cases. Detection of urinary hepcidin-25 level in association with other iron profile markers in large prospective studies could strengthen our results.

Conclusions

Hepcidin can be useful investigation in diagnosing ID in early stages in apparently healthy young females, so that the timely intervention strategies can be started to reduce morbidity. Further larger multicentered prospective study will be necessary to confirm these findings.

Ethical Approval

The study was conducted after getting ethical approval from Medical Ethics Committee of SGT university, Gurgaon, Haryana, India.

Shree Guru Gobind Singh Tricentenary University (SGT University), The Faculty of Medicine and Health Sciences (FMHS).

Ethical clearance Reference No: SGTU/FMHS/D./96, Dated March 14, 2016.

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Contribution Details

All the authors have substantial contributions to conception and design, acquisition, analysis, interpretation of data, drafting the article, revising it critically, and final approval of the version to be published.

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Conflict of Interest

All authors declare that they do not have any conflict of interest.

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