Validity of Serum And Urinary Hepcidin As Biomarkers of Late-Onset Sepsis of Premature Infants

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Abstract

Background: Sepsis remains one of the leading causes of neonatal morbidity and mortality particularly among premature infants. Blood culture is the “gold standard” for diagnosis of neonatal sepsis but is associated with several pitfalls. Adjunctive diagnostic tests, including biological markers should be used to aid in antibiotic -starting decision in presumed septic preemies until culture results are available

Aim of the work: We aim to evaluate the validity of measuring serum hepcidin concentration as diagnostic biomarker for late-onset sepsis in preemies and to quantify its cut-off value that differentiate truly septic from non-septic symptomatic premature infants. In addition, to examine the correlation between serum hepcidin (Hep-S) and urinary hepcidin (Hep-U) and to find out if measuring Hep-U can be used as an alternative safe, non-invasive biomarker for late-onset sepsis diagnosis, without exposure to frequent phlebotomy and its risks.

Patients and Methods: The current case-control study included seventy-three (73) cases of clinically and laboratory confirmed late-onset sepsis as "case group" and fifty (50) non-septic premature infants of comparable age and gender as "control group". All participants were evaluated as per unit protocol to rule out sepsis by complete blood count, CRP, blood, urine, CSF and other cultures as indicated, plus different radiologic modalities as needed. Acute serum and urinary hepcidin concentration were evaluated by ELISA for all participants at enrollment "acute sample". After one week of treatment, convalescent samples for serum and urine hepcidin were collected and compared with the acute samples.

Results: Statistically significant higher concentration of both serum and urinary hepcidin were recorded among cases as compared with non-septic peers ($t=44.2$ & $p=0.0001$, $t=23.8$ & $p=0.0001$ for serum and urine hepcidin respectively). Similarly, Significant reduction of hepcidin at different body fluids was recoded after one week of treatment as compared with acute samples (paired $t=18.1$ & $p=0.001$, paired $t=14.1$ & $p=0.001$ for serum and urine hepcidin respectively). Significant direct correlations were reported between acute serum hepcidin levels and CRP, urinary hepcidin, and total leucocyte count. While significant negative correlation was recorded with platelets count. AUC of serum hepcidin ROC is 0.93, A cut off value of ≥ 94.8 ng/ml of S. hepcidin showed sensitivity (88%), specificity (94%), PPV (95%) and NPV (84%) respectively with accurately diagnosing 90.2% of presenting cases as septic or not. While urinary hepcidin showed slightly less discriminating ability with AUC of 0.87. At cut-off value of ≥ 264 ng/mg of urinary hepcidin/urinary creatinine showed sensitivity (85%), specificity (90%), PPV (92.5%) and NPV (81%) respectively with accurately diagnosing 84.5% of presenting cases as septic or not.

Conclusions: Hepcidin concentration in different body fluid can function as promising accurate and rapid surrogate test, with blood culture, that guide empiric antibiotics – starting decision or withholding it safely until the culture results is ready in symptomatic presumed septic preemies. Urinary hepcidin has advantages over serum hepcidin as; it is non-invasive, no hazards of phlebotomy, and less variable throughout the day.

Introduction

Sepsis remains one of the leading causes of neonatal morbidity and mortality particularly among premature infants(1). It ranks as the third cause of neonatal death following prematurity and intrapartum associated complications(2). It affects an estimated three million neonates worldwide with a mortality of 11%-19% and unquantified long-term neurological deficits(3). Premature infants have three to ten times higher risk of developing late-onset sepsis (LOS) than full term infants due to low level of maternal IgG, and exposure to invasive procedures at NICU setting(4). Classification of neonatal sepsis (N.sepsis) based on the time of onset of symptoms helps to guide empiric antibiotic therapy as it implies difference in the presumed mode of transmission and predominant causative organisms(5). Clinical manifestations of neonatal sepsis (N.sepsis) are non-specific ranges from “not doing well” to severe focal or systemic disease(6).

Timely diagnosis and prompt initiation of antimicrobials is paramount to mitigate the deleterious effects of sepsis and its associated morbidities, and high case fatality(7). The vexing challenge in managing neonatal sepsis is the inability to differentiate truly septic from non-septic infants by rapid accurate test (8). Blood culture is the “gold standard” for diagnosis of N. sepsis, but is associated with several pitfalls; first, is the long turn-around time for results even with the updated techniques (9), second is the high false negative results due to small inoculum size and intermittent nature of bacteremia(10). Accordingly, adjunctive diagnostic tests including hematologic indices, scoring system and biological markers should be used to aid in antibiotic -starting decision in presumed septic preemies until culture results are available (11)

Hepcidin is a 25-amino acid peptide that is produced by the liver (Hep) (12),and proved to have bactericidal properties (cidal), and accordingly labelled as HEPCIDIN(13). It is well reputed as negative feedback master regulator of iron hemostasis in human(14). Hepcidin lowers serum iron by mediating iron sequestration inside enterocyte, macrophage, and monocyte through downregulation of ferroportin expression on iron export channels(12). Hepcidin-ferroportin complex leads to internalization and degeneration of ferroportin within the cells with subsequent reduction in extracellular iron(15) As iron is an essential element for both host and pathogen, hepcidin may plays an important role in combating infection through hypoferremia that impair bacterial survival(16). Moreover, it is documented that additional role of hepcidin in innate immunity is by altering macrophage immune response involving cytokine and nitric oxide production(17). In vitro studies described hepcidin as having direct antibacterial and antifungal effects(18). Hepcidin expression is induced by IL-6 and it's urinary excretion is increased 7.5 folds within 2 hours of IL-6 infusion(14)

Twenty and 22 amino acid metabolites of hepcidin exist in urine (19).however, Only 5% of hepcidin that filtered by the kidney is found in urine. This is explained by reabsorption and degradation of hepcidin in proximal tubules similar to other small peptides(20)

The aim of the current work is to evaluate the validity of measuring serum hepcidin as diagnostic biomarker for late-onset sepsis in preemies and to quantify its cut-off value that differentiate truly septic

from non-septic symptomatic premature infants. In addition, to examine the correlation between serum hepcidin (Hep-S) and urinary hepcidin (Hep-U) and to find out if measuring Hep-U can be used as an alternative safe, non-invasive biomarker for late-onset sepsis diagnosis without exposure to phlebotomy and its risks.

Patients And Methods

The current case-control study was carried out in neonatal intensive care unit (NICU) of Zagazig University Children Hospital-Egypt during the period from July 2019 to December 2020. Our NICU is a referral center for almost 20 small towns and villages around the city, it is equipped with 25 incubators supplemented with phototherapy, mechanical ventilators, bubble CPAC, Heated Humidified high flow nasal cannula (HHHFC) as needed. Qualified neonatologist and registered nurses are available around the clock in our unit.

Patients:

Two hundred seventy- four (274) preterm neonates were evaluated for clinically suspected late-onset sepsis during the study period. They presented with variable and non-specific manifestations. All were subjected to thorough history taking, full clinical evaluation and routine sepsis work up as per unit protocol. Only 102 neonates fulfilled "proven sepsis" criteria both clinically and laboratory, of them twenty three (23) neonates who had significant anemia and/or recent history of blood transfusion, within the previous 2 weeks, had been excluded from the study. The remaining seventy-nine (79) cases represented the **"case" group** and 50 non-septic (asymptomatic, negative cultures) preterm infants with comparable demographic characteristics were included as **"control" group**. Unfortunately, 6 extremely- low birth weight mechanically ventilated infants succumbed before obtaining the convalescent samples. Seventy-three (73) infants completed the study, and their data were analyzed accordingly, as displayed in the study flow chart (Fig-1).

Exclusion criteria: Term infants, premature babies who presented with suspected sepsis earlier than seven days old, premature infants with significant anemia or history of recent transfusion, clinically suspected septic premature but with negative culture, and also infants with multiple congenital anomalies have been excluded from the study.

Definitions:

Late-onset sepsis: describes cases presenting after the seventh day of life(21)

Suspected sepsis: clinical and non-specific laboratory finding of sepsis are present, but the failure to show the causative microorganism in cultures (22)

Proven sepsis: clinical and laboratory finding are present, and demonstration of the pathogenic causative microorganism in the cultures taken from sterile fields(22)

Methods:

All presented cases were subjected to sepsis work-up as per unit protocol, it includes: complete blood count, C-reactive protein, blood culture, urine and CSF analysis and culture, variable radiological modalities as X-ray, ultrasonography as needed.

All participants (control and cases) were subjected to evaluation of serum hepcidin levels (Hep-S), for cases; two samples are evaluated, one at initial presentation (acute sample) and the second at seventh (7) day after starting treatment (convalescent samples). while for healthy control, only single sample at presentation was evaluated. Concurrent measurement of urinary hepcidin levels (Hep-U) for all participants, at the same phases, were also carried out.

Serum and urinary hepcidin: The concentrations of 25- amino acid hepcidin in serum and urine were determined using a double antibody sandwich ELISA (kits provided Cusabio Life Science; China, kit number CSB-E13026h) according to the manufacturer's instructions. As hepcidin is polypeptide consists of 25 amino acids so it is very fragile and labial to proteases, accordingly, both blood and urine samples were collected in EDTA tube containing proteases inhibitor (aprotinin) to prevent coagulation and action of proteases. To avoid frequent phlebotomy and invasive maneuvers, acute serum sample was taken at the same time of initial culture, centrifuged and stored at -80 for batch analysis. Similarly acute urine samples were taken, by catheter or suprapubic aspirate at the same time of urine evaluation as part of late-onset sepsis work-up (23) while convalescent sample is taken by first void in urine bag collector after taking convalescent serum sample, all are stored at -80c. Urinary creatinine was also measured in urine samples by the Jaffe reaction using the creatinine parameter assay (R&D systems, Minneapolis, MN) to evaluate urinary hepcidin /creatinine concentration that expressed as ng/mg.

This research has been approved by Institutional Review Board (IRB) of Zagazig University and informed consent was taken from parents/ or legal guardian of each child before enrollment in the study.

Statistical analysis:

Data were statistically analyzed using SPSS program (Statistical Package for Social Science) version 22.0 (SPSS Inc., 2007). Qualitative (categorical) data were represented as frequencies and percentages. Chi-Square test (χ^2) test was carried out for testing the association between the qualitative data frequencies. Quantitative (numerical) data were represented as mean and standard deviation (SD) and student's t-test was used to detect difference between groups, which were normally distributed. Nonparametric variables were presented as median & inter quartile ranges (IQRs) and Mann-Whitney test was used to detect the difference between these groups. To assess the presence of significant dynamic changes of serum, urinary hepcidin and CRP after seven days of treatment as compared with acute concentrations, paired t test was performed to determine this issue. The Pearson correlation coefficients were used to estimate association between acute serum hepcidin with clinical and laboratory data namely CRP, urinary hepcidin, and different hematological indices. In order to assess the utility of serum and urinary hepcidin as biomarkers for neonatal sepsis diagnosis, we compared the areas under receiver

operating characteristic (ROC) curves between hepcidin values and blood culture. Several clinical and laboratory parameters were used in constructing prediction model for late-onset sepsis using logistic regression analysis and the results were reported as odds ratio (OR) with 95% CI. The test results were considered significant when $p\text{-value} \leq 0.05$, all p values are two-tailed.

Results

A total of one hundred and twenty –three (123) premature infants were involved in the current study, as seventy-three (73) cases with clinical and laboratory proven late-onset sepsis "case group" and fifty (50) non-septic premature infants of comparable age and gender as "control group". 72 (58.5%) of them were male, majority of them had gestational age (GA) ranges from 28-35 weeks (60 infants -48%), while 32 % (39 infants) were late preterm (GA >35 weeks). All participants of the case group were hospitalized at enrollment to the study (65% still at hospital since birth, 35% readmitted after developing symptoms) while majority (35-70%) of the selected controls were discharged from hospital and are following up regularly at neonatal outpatient clinic. Cases were screened to rule- out late onset sepsis at mean age of 18.6 ± 2.3 days versus 19 ± 1.9 days for controls' enrollment at the study. No statistically significant difference could be detected between participants at case and control groups regarding age at presentation, gestational age, birth weight, or gender (**Table-1**). Majority of preterm infants needed respiratory support ranges from low flow nasal cannula (LFNC) to mechanical ventilator, cases need much more intensive support than controls. 18% of septic premature infants were on mechanical ventilation compared with only 6% of non-septic comparable ($p=0.05$). Forty (80%) non-septic preterm are feeding enterally while 63% of septic cases are on parenteral nutrition ($p=0.0001$). None of enrolled infants received erythropoietin, however, as per unit protocol, any infant who reached full enteral feeding, is supplemented with iron as shown in **Table-1**.

Leading isolated causative pathogens among our septic preemies were gram negative organisms [Klebsilla pneumonia (47%) & pseudomonas (6%), citrobacter (6%)] followed by several subtypes of staphylococcus [staphylococcus hemolyticus (20%), staphylococcus hominus (15%), staphylococcus warneri 6%]

Complete blood count for septic preemies were associated with significantly; higher total leucocyte count, increased percentage of those with immature/total ratio of >0.20 , higher rates of neutropenia than non-septic peers. On the contrary, they had significantly lower hemoglobin level and platelets count as compared with non-septic participants (**Table-2**).

C reactive protein (CRP), serum and urinary hepcidin were significantly higher among septic premature infants as compared with non-septic participants. Convalescent samples that taken from septic cases one week after starting treatment showed significantly lower CRP, serum and urinary hepcidin as displayed in **Table- 2**. Two extreme preterm infants had progressive increased levels of hepcidin at different body fluid, associated with worsening of their clinical status.

Pearson's correlation was examined between acute serum hepcidin concentrations and relevant quantitative clinical and laboratory variables as displayed in **Table -3**.

Significant direct correlations were recognized with CRP ($r=0.23$ $p=0.03$), acute urinary hepcidin ($r=0.42$ $p=0.01$), and total leucocyte count. While significant negative correlation was recorded with platelets count. No correlation could be detected with birth weight or gestational age. In addition, correlation is maintained between serum and urinary hepcidin after one week of treatment, convalescent sample ($r=0.35$ $p=0.023$) as displayed in scatter plots (Figures 2 & 3).

For evaluating the discriminating power of measuring serum and urinary hepcidin in correctly classifying septic from non-septic infants, receiver operator curves (ROC) were constructed and area under the curve (AUC) was determined for each as reported in **Table-4 and Figures 4 & 5**.

Highly significant discriminating ability with AUC of serum hepcidin ROC is 0.93. At cut off value of ≥ 94.8 ng/ml of S. hepcidin showed sensitivity (88%), specificity (94%), PPV (95%) and NPV (84%) respectively with accurately diagnosing 90.2% of presenting cases as septic or not. While urinary hepcidin showed slightly less discriminating ability with AUC of 0.87. At cut-off value of ≥ 264 ng/mg of urinary hepcidin/urinary creatinine showed sensitivity (85%), specificity (90%), PPV (92.5%) and NPV (81%) respectively with accurately diagnosing 84.5% of presenting cases as septic or not.

Prediction model for neonatal sepsis was calculated by logistic regression analysis to find out real predictors of late-onset sepsis of premature, high CRP and elevated hepcidin concentration in both serum and urine were reliable predictors as shown in **Table -5**.

Discussion

Sepsis represents a major contributor to neonatal mortality particularly among premature infants (24) with mortality rates as high as 40% in VLBW(25). Adaption of universal intrapartum antibiotic prophylaxis (IAP) has resulted in 80% reduction of early-onset sepsis cases, but incidence of late-onset sepsis is significantly increased in modern NICU due to increased survival of extreme preemies with many invasive strategies to support their life(26)(4). Frequent screening for late-onset sepsis, with any change in their clinical status, is an essential strategy for early diagnosis and management of infection as early as possible (27). Clinical diagnosis is not feasible due to the non-specific nature of sepsis manifestations (28). Although blood culture is the cornerstone for sepsis diagnosis, it is associated with several drawbacks that renders it not suitable for rapid diagnosis or guiding antibiotic- starting decision. Critically ill septic premature infants may succumb before the result of culture is available(16). Also, the high rate of false negative results is another important challenge for the use of blood culture solely for sepsis diagnosis (29). In our study only 37% (102/274) of evaluated cohort for late-onset sepsis (LOS) had positive culture, similarly **Betty and Inderpreet** reported 51% culture positive rate among clinically presumed septic neonates(30). The risk of morbidities and mortality due to delayed or untreated infection versus the short and long-term hazards of frequent un-needed exposure to antibiotics, justify the pressed need for accurate and rapid test for differentiating septic from non-septic symptomatic premature infant.

In the current research we hypothesized that, as hepcidin is a well-known first line defender that rapidly increases to militate against pathogenic invasion (16), (17), its measurement can point to the septic etiology of non-specific manifestations of neonate. Coincide with our assumption, statistically significant higher serum hepcidin (Hep-S) values were recorded for culture- proven septic premature infants in our cohort as compared with their non-septic asymptomatic peers. Similarly, **Wu and his team** affirmed that infection is a potent inducer of hepcidin production in VLBW as it increases four folds in newborn with proven infection (9). Other researchers examined the usefulness of hepcidin level's measurement in cord blood and in serum as a biomarker of early onset sepsis and described it as valid predictor (7)(31). Serum hepcidin (Hep-S) was dramatically elevated in infants who are bacteremic or even treated for presumed sepsis but with negative culture in previous report (18). Infection and inflammation enhance IL-6 production which in turn induces hepatic hepcidin production to combat pathogenic invasion. Also, autocrine synthesis of hepcidin, can more selectively activate the appropriate cell type namely macrophages and monocyte that can fine tune the response to infection (32). Hepcidin fights pathogen either by depriving it from iron (33) or by direct antimicrobial activity against various pathogen(34). In follow-up of our septic cohort, serum hepcidin (Hep-S) were significantly lower after seven days of antibiotic therapy (convalescent sample) as compared with the acute sample, denoting the parallel dynamics of hepcidin with infection and inflammation. It is worthy note that 2 mechanically ventilated extreme premature infants in our cohort showed progressive worsening of their clinical condition despite several trials of antibiotics, both of them had progressive increase in acute phase reactants and also in serum and urinary hepcidin and they died few days after taking convalescent samples. Their cultures came positive for multidrug resistant klebsiella pneumonia. This finding is a supporting evidence for the prognostic value of hepcidin, nevertheless, this issue needs to be thoroughly investigated. We excluded the anemic and recently transfused preemies to evaluate the changes of hepcidin with variable stages of infection without other interfering factors especially those related to iron hemostasis. Receiver-operator curve for hepcidin showed AUC of 0.93 that indicates high discriminating ability of serum hepcidin concentration in differentiating septic from non-septic infants. As compared with several commonly used acute phase reactant such as CRP and procalcitonin which have good negative predictive values (NPV) but low positive predictive values (PPV) to diagnose cases of sepsis correctly (35), hepcidin showed high diagnostic profile. A cut-off value of ≥ 94.8 ng/ml of S. hepcidin showed sensitivity (88%), specificity (94%), PPV (95%) and NPV (84%) respectively with accurately diagnosing 90.2% of presenting cases as septic or not. Close to our findings, hepcidin was reported to correctly diagnosing sepsis among both term and preterm infants by 91% at cut-off of ≥ 92 ng/ml (9). Acute serum hepcidin values showed weak positive correlation with CRP levels ($r=0.2$ $p=0.03$). Despite the fact that, production of both of them are IL-6 dependent, (32)(36), lack of strong correlation between both of them may be explained by the different time course of their production. CRP is delayed for 48 hours to peak and its half-life time is approximately 24 hours(37), while hepcidin peaks in few hours (6 hours) (14).

Serum hepcidin levels at initial evaluation (acute) and after one week of treatment (convalescent) displayed significant positive correlation with corresponding urinary hepcidin values ($r=0.42$, $r= 0.35$ respectively). This finding may answer our question "Could we find body fluid to diagnose and follow up

sick preemies, other than blood?" to avoid frequent phlebotomy and its hazards. As hepcidin is excreted and can be accurately quantified in urine sample by same analytic method (ELISA) as serum hepcidin, and with this positive correlation, urinary hepcidin may replace serum hepcidin in initial diagnosis and follow up of septic preemies, particularly if frequent screening is needed during their NICU stay. Hep-U is significantly higher among clinically and laboratory proven sepsis cases as compared with non-septic infants in our series, and its level is significantly reduced with improved cases after 7 days of treatment. All previous data support the idea that, similar to Hep-S, urinary hepcidin is dramatically increased in response to IL-6 infusion(14) and its quantification can help in rapid, accurate diagnosis of sepsis in premature infants. In harmony with our data, researchers in pediatrics and adult documented the positive serum and urinary hepcidin correlation (38) (20). Simultaneous adult samples usually show better correlation than those in premature as urine sample may be delayed until involuntary void in urine bag after taking serum sample. As hepcidin follows diurnal variation(20), such delay may impair correlation.

Area under the curve of urinary hepcidin receiver operator curve is 0.87, indicating good discriminating power of measuring urinary hepcidin concentration between septic and non-septic. At cut-off value of ≥ 264 ng/mg of urinary hepcidin/urinary creatinine showed sensitivity (85%), specificity (90%), PPV (92.5%) and NPV (81%) respectively with accurately diagnosing 84.5% of presenting cases as septic or not. Urinary hepcidin was normalized for urinary creatinine, and expressed as ng/mg, to adjust for variable urine dilution. Diagnostic profile for urinary hepcidin is reasonable, with less diagnostic power than those of S. hepcidin but still with accepted accuracy in sepsis diagnosis. Up to our knowledge, no previous study was performed to evaluate the role of urinary hepcidin in sepsis diagnosis either in children or neonates. However, Muller and colleagues reported positive correlation between Hep-S and Hep-U and between both of them with iron indices(38). Accordingly, Researchers were hoping to use Hep-U as diagnostic and prognostic biomarker for iron deficiency anemia, and their results were promising(39). Another advantage of using Hep-U as diagnostic biomarker for N.sepsis, added to its non-invasive nature, is the less impact of diurnal variation on its level(40)

Although hepcidin has been discovered as early as 2000, its clinical application was delayed due to the expensive, cumbersome and invasive nature of its measuring modalities like hepcidin mRNA expression in liver biopsy sample. In 2008, enzyme linked immunosorbent assay for human hepcidin is developed that can accurately, easily and reproducibly determine physiologic and pathologic changes in hepcidin in variable body fluids(20).

Significant proportion of our cases were late preterm or near-term infants (39 infants, 32%) with gestational age 35-37 weeks. Usually those infants are apparently well with safe pulmonary status, thermal control and nutritional abilities, but indeed they significantly have issues of prematurity like poor immunity, increased risk of feeding problems, and injurious hyperbilirubinemia(41). These criteria necessitate their close follow-up to avoid such hazards.

Leading isolated causative pathogen among our septic preemies is gram negative organisms [Klebsilla pneumonia (47%) & Pseudomonas (6%), Citrobacter (6%)] followed by several subtypes of

staphylococcus. Previous study from other Egyptian children hospital, showed the same results (42). On the other hand, coagulase negative staphylococcus is the most common isolates of late-onset sepsis of premature in several developed countries (53%-78%) and (35%-47%) in series from developing countries. The predominant causative pathogen is affected by the geographic areas, and level of health care service, particularly adherence to infection control strategies(43)

All the previous data conclude that hepcidin concentration in different body fluid can function as promising accurate and rapid surrogate test, with blood culture, that guide empiric antibiotics –starting decision or withholding it safely until the culture results is ready in symptomatic presumed septic preemies. Urinary hepcidin has advantages over serum hepcidin as; it is non-invasive, no hazards of phlebotomy, and less variable throughout the day.

Our study has some limitation; first, small number of involved premature infants in case group as inclusion criteria was strict to involve only culture- proven cases who presented almost one third of screened cases. Second, the dual function of hepcidin as master iron regulator plus its role in innate immunity forced us to exclude all anemic or recently transfused cases to prevent bias due to disturbed iron status. Majority of extreme premature infants suffer from anemia or receive transfusions somewhere during their NICU stay and thus hepcidin may not be the ideal biomarker for their screening. To widespread its use, especially urinary hepcidin, wide-scale multicenter study should be performed that involve anemic, transfused, and culture negative cases to determine diagnostic cut-off of hepcidin in different situations.

Declarations

- Ethical Approval and Consent to participate: the study was approved by the research and ethical committee of the participating hospitals. All parents of enrolled children signed written informed consents for participation of their children in the current study.
- Consent for publication: All parents of enrolled children signed written informed consents for publication the current study.
- Availability of data and materials: all data and materials related to the study are included in the current manuscript.
- Competing interests: all authors declare no competing interests related to the study
- Funding: No funds were available for the current research
- Authors' contributions:

HS, HM: set the idea of the study and designed the study.

HS, NK: reviewed literature, drafted the manuscript, critically analyzed the data.

HS, HM, MA: collected data

AE: performed the laboratory testing

All authors reviewed and approved the manuscript for final publication

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Tables

Table-1: Demographic and clinical characteristics of the studied group

Variable	Case N(73)	Control N(50)	Test	P value
Gestational Age(week)				
<28 N(%)	15(21)	9(18)	X² 0.42	0.51
28-35 N(%)	34(47)	26(52)		
>35 N(%)	24(32)	15(30)		
Mean ±SD	32.7±3.1	33.6±3.2	t 1.5	0.12
Age at enrollment (days)	18.6±2.3	19.1±1.9	t 1.26	0.20
Birth weight (grams)	1690.1 ±356	1761.7±374	t 1.00	0.28
Gender				
Male N(%)	42(57)	30(60)	X² 0.10	0.074
Female N(%)	31(43)	20(40)		
Hospitalization at presentation				
Yes N(%)	48(65)	15(30)	X² 14.4	0.001
No N(%)	25(35)	35(70)		
Hospital Stay (days)				
Mean ±SD	24	10	MW 6.7	0.0001
Range	18-37.5	7.5-11		
Respiratory Support				
Room air N(%)	0	37(74)	X² 0.65	0.053
Head box N(%)	10(14)	0		
Nasal cannula (LFNC) N(%)	30(41)	5(10)		
HHHFNC N(%)	9(12)	0		
NCPAP N(%)	11(15)	5(10)		
Mechanical Ventilation N(%)	13(18)	3(6)		
Nutritional Support				
Enteral N(%)	27(37)	40(80)	21	0.0001
Parenteral N(%)	46(63)	10(20)		
Iron Therapy N(%)	0	0		

Yes	32(43)	43(86)	22	0.0001
No	41(57)	7		
Erythropoietin N(%)	0	0		

N number, % percent, SD standard deviation, t student t test, X^2 chi-square, HHHNC high humidified heated nasal cannula

Table-2: Laboratory finding of the studied groups

Variable	Case N(73)	Control N(50)	Test	P value
Total leucocytic count	16.6*10 ³	10.6*10 ³	MW4.04	0.0001
Median (IQR)	(11.2-19.4)	(7.3-15)		
Neutropenia N(%)	22(29)	3(6)	X ² 10	0.0014
Immature/mature >0.2 N(%)	65(88)	6(11)	X ² 56	0.0001
Hematocrit				
Mean ±SD	47.4±7.7	50.7±8.5	t 1.73	0.08
Range	(36-61)	(39-65)		
Platelet's count				
Mean ±SD	101±20.5*10 ⁶	273±30*10 ⁶	t 13.01	0.001
Range	(50-130)	(112-540)		
Acute CRP (mg)				
Mean ±SD	56±16.5	2.7±1.5	t 19.1	0.0001
Range	(25-97)	(1-5)		
Convalescent CRP (mg)			t* 12	
Mean ±SD	33.5±27	-		0.001
Range	(7.1-69)			
Acute serum Hepcidin(ng/ml)				
Mean ±SD	288±34	66.0±12	t 44.2	0.0001
Range	(180-460)	(42-94)		
Convalescent serum Hepcidin(ng/ml)				
Mean ±SD				
Range	98.3±31 (42-150)		t* 18.5	0.0001
Acute urinary Hepcidin(ng/ml)				
Mean ±SD	422±65	190±27	t 23.8	
Range	(322-580)	(127-260)		0.0001
Convalescent urinary Hepcidin(ng/ml)				
Mean ±SD	231±56			

Range	(150-400)	t* 14	0.0001
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N number, % percent, SD standard deviation, MW Mann Whitney test, t student t test, X² chi-square, t * paired t test, between acute and convalescent sample of the same variable.

Table-3: Pearson's correlations between acute serum hepcidin level and clinical and laboratory data of studied premature infants (n=123).

Variable	r	p value
Gestational age	- 0.1	0.42
Birth weight	0.069	0.58
Hospital stay	0.417	< 0.0001
Laboratory measurements		
• Acute CRP	0.633	< 0.0001
• TLC	0.402	0.001
• Hb level	0.033	0.79
• HCT	0.033	0.79
• Platelets count	-0.042	0.74
• Acute urinary hepcidin	-0.279	0.021
	0.41	0.017
Patient outcome	0.185	0.13

TLC, total leucocytic count, HCT hematocrit, r correlation coefficient, CRP C-reactive protein, Hb hemoglobin

Table-4: Discriminating value of serum and urinary Hepcidin

parameter	Serum hepcidin	Urinary Hepcidin
AUC	0.935	0.878
Standard Error	0.028	0.040
Significance (p)	<0.0001	<0.0001
95% confidence interval CI	0.879-0.991	0.80-0.956
Parameter	Serum hepcidin ≥94.8ng/ml	Urinary Hepcidin ≥264 ng/mg
Sensitivity	88%(64/73)	85% (62/73)
Specificity	94% (47/50)	90%(45/50)
Positive predictive	95% (64/67)	92.5%(62/67)
Negative Predictive	84% (47/56)	81%(45/56)
Accuracy	90.2%(64+47/123)	84.5%(62+42/123)

Table (5): Logistic regression analysis for predictors of neonatal sepsis

Independent variables	S.E	Beta	Wald	p value	95% C.I.	
					Lower	Upper
- Birth weight	.014	.065	1.637	0.107	-.005	.052
- Gestational age	.000	-.055	-1.338	0.186	.000	.000
• Urinary hepcidin	.000	.179	2.625	0.011*	.000	.001
• Sex	.147	.931	.025	0.874	0.187	7.185
• serum hepcidin	.000	.400	7.006	<0.0001**	.001	.002
• CRP	.001	.316	4.998	<0.0001**	.003	.008
- Constant	-41.589	13.044	10.166	0.001		

Figures

Figure 1

Flow chart of the study

Figure 2

Scatter diagram showing significant direct correlation between acute serum and urine hepcidin ($r, 0.4$ & $P, 0.01$)

Figure 3

Scatter diagram showing significant direct correlation between convalescent serum and urine hepcidin ($r, 0.35$ & $P, 0.023$)

Serum hepcidin ROC Curve

Figure 4

Receiver operating characteristic curve (ROC) for serum hepcidin

Figure 5

Receiver operating characteristic curve (ROC) for urinary hepcidin

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