Assessment of Urinary Interleukin-18 and Serum Amyloid A Efficacies against C-Reactive Protein in Diagnosis and Follow-up of Neonatal Sepsis

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Abstract

Objective: infection is a leading cause of morbidity and mortality in neonates. The aim of this study was to evaluate the diagnostic and prognostic performances of urinary interleukin-18 (uIL-18) and serum amyloid A (SAA) in neonatal sepsis parallel to C-reactive protein (CRP).

Subjects and methods: A total of 275 neonates were included in this case-control study. This study was conducted in neonatal intensive care unit at both Minia University Hospital for obstetric and pediatrics as well as Qena University hospital (Egypt). Among those 275 neonates, 150 non septic neonates - who had neither clinical signs nor laboratory findings suggestive of sepsis - were involved as a non-septic control group (Group II) and 125 septic neonates were classified as a septic group (group I). Blood and urine samples were obtained before initiation of antibiotic therapy. Full sepsis screen was performed at the time of sepsis onset plus measurement of SAA and uIL-18 by Enzyme Immune Assay (EIA). A second blood and urine samples were collected 72 hours later. The effectiveness of these 3 biomarkers as diagnostic ones was determined by using receiver operating characteristic (ROC) curve analysis. We also calculated the sensitivity, specificity, and positive, and negative predictive values.

Results: The levels of uIL-18 and SAA were significantly elevated in septic neonates than in control group. The two markers showed significant decrease in their levels after 72 hours which matched with clinical improvement but not CRP. Moreover, very high levels of these markers were observed in neonates who died later on. The area under ROC curve (AUC) was used to evaluate the diagnostic efficacies of these markers. The superiority of SAA over both uIL-18 and CRP was obvious in the diagnosis of neonatal sepsis [AUC: 0.995] versus [AUC: 0.934 (uIL-18) and 0.871 (CRP)]. However in discriminating late onset sepsis (LOS), both uIL-18 and SAA have equal diagnostic capacities [AUC: 0.991] which are better than that of CRP [AUC: 0.866]. As to early onset sepsis (EOS), SAA has the most efficient performance but CRP is advanced than uIL-18. The specificities and sensitivities of either SAA or uIL-18 were higher than that of CRP in differentiating neonatal sepsis mainly LOS from controls.

Conclusion: Both uIL-18 and SAA have enhanced performance over CRP for distinguishing neonatal sepsis mostly for LOS. Thus, they could be promising biomarkers for the screening and follow up of neonatal sepsis. This warrants further assessment of their prognostic values.

Introduction

Neonatal Sepsis is a systemic inflammatory response that mainly results from bacterial infection in the first month of life [1]. Neonatal sepsis is one of the most serious and major causes of morbidity and mortality in new-borns [2]. It is a life-threatening event affecting 3–5 neonates per 1000 live-births [3]. Sepsis is ranked as the sixth leading cause of death among neonates and the eighth leading cause of death for infants through the first year of life [4]. The World Health Organization (WHO) estimates that 1 million deaths per year (10% of all under-five mortality) are due to neonatal sepsis and that 42% of these deaths occur in the first week of life [5].

Neonates are unable to respond early to infection because of immunologic deficiencies. Additionally, concomitant conditions often make it difficult to diagnose and hence manage the neonatal sepsis. Also, the clinical manifestations of newborn infections vary [6]. Accordingly, the early precise diagnosis of neonatal sepsis is usually missed and as neonatal sepsis is a severe global problem hence there is a crucial need for reliable biomarkers to differentiate between early infected and non-infected new-borns.

Even though blood culture has been considered the most reliable test for the diagnosis of sepsis but it is too slow and has false negative outcomes. As well, C-reactive protein (CRP) is the most commonly used marker for the diagnosis and follow up of sepsis that clinicians depend on it in all hospitals. It has a low specificity and comprises a physiological 3-days increase resulting in a low possibility to detect sepsis at an early stage [7]. Thus, to date a single biomarker is not sufficiently reliable for diagnosis of neonatal sepsis. Consequently, it is necessary for us to focus on the combination of different biomarkers in hope to achieve definite conclusions.

Cytokines are regulators of the immune response that have a role in the pathophysiology of sepsis [8]. Interleukin-18 (IL-18) is a member of the Interleukin-1 cytokine super-family which plays an important role in regulating immune responses [9,10]. It is a unique cytokine with a capability to induce either T helper 1 or 2 polarization depending on the immunologic context [11]. Some studies have found that the level of urinary IL-18 (uIL-18) may be influenced by the presence of sepsis in critically ill adults, children and neonates suggesting a significant association between the urinary biomarkers and systemic inflammation as well as sepsis [12-14].

Serum Amyloid A (SAA) is an acute phase reactant regulated by pro-inflammatory cytokines (interleukin-6 and tumor necrosis factor-alpha). Liver, smooth muscles, macrophages, adipocytes and endothelial cells are involved in its production. Multiple functions such as chemotaxis, immunomodulation and tissue regeneration have been attributed to SAA [15,16]. A 1000-fold increase in the concentration of SAA has been reported during neonatal sepsis [16].

Both uIL-18 and SAA have been suggested as biomarkers for neonatal sepsis [12,17]. Yet, no studies have explored the value of uIL-18 along with SAA in the diagnosis and follow up of neonatal sepsis. Furthermore, either too small or none adequately powered clinical studies exist regarding the comparison between these two markers and CRP as being the established conventional marker for neonatal sepsis [6]. Accordingly, we tested the value of uIL-18 and SAA in the diagnosis as well as follow up of neonatal sepsis compared to the levels of CRP. As well, our goal was to evaluate the diagnostic outcomes of both uIL-18 and SAA along with CRP in identifying septic neonates.

Subjects and Methods

Subjects

This study included almost all neonates admitted at neonatal intensive care unit (NICU) from both Minia University Hospital for obstetric and pediatrics as well as Qena University hospital during the period from May 2013 to April 2015. A number of neonates were not drawn in because of failure in collecting urine or blood sample at the time of clinical deterioration. As well, the neonates who fulfilled the exclusion criteria of this study (mentioned below) or those who had unanticipated discharge from NICUs were not included. A total of 275 neonates were involved in this case-control study. Among those 275 neonates, 150 non septic new-borns that had no clinical signs or laboratory findings suggestive of sepsis were involved as a control group (Group II). The septic neonates (group I) consisted of 125 neonates who were recruited as they developed acute clinical deterioration and/or laboratory findings suggestive of sepsis. This patient's group was further subdivided into two subgroups as early-onset neonatal sepsis (EOS) group Ia and late onset neonatal sepsis (LOS) group Ib. EOS subgroup involved 58 neonates with the onset of septic diagnostic criteria within the first 72 hours of their life while LOS subgroup consisted of 67 neonates who developed sepsis late after the first 72 hours of birth [18]. Moreover, those neonates were followed up after the next 72 hours from the onset of sepsis concerning their clinical signs and laboratory findings. Neonates with severe life threatening congenital malformations, hypoxic ischemic encephalopathy, intracranial hemorrhage or who developed acute kidney injury (AKI) were excluded from the study. AKI is a rapidly developing renal dysfunction. AKI was defined as an increase in serum creatinine level by 0.3 mg/dl and/or 50% from baseline or reduction in urine output to <0.5 mL/kg/hour for 6 hours [19,20].

Diagnosis of sepsis was done according to the 2001 International Sepsis Definitions Conference criteria [21]. The clinical criteria taken as indicative of sepsis include temperature instability (hypothermia/hyperthermia), apnea/tachypnea/cyanosis/respiratory distress or need for mechanical ventilation, tachycardia/bradycardia, hypotension/poor perfusion, irritability/lethargy, feeding intolerance, and abdominal distension/hepatosplenomegaly/jaundice [22,23]. Laboratory tests used for sepsis screen consisted of white blood cells (WBCs) count, absolute neutrophil count (ANC), and CRP. Abnormal results in two or more of these tests were supportive for the diagnosis of infection [23]. The results were considered abnormal if WBCs count<5,000/mm³ or >25,000/mm³ at birth, >30,000/mm³ at 12 to 24 hour or >21,000/mm³ after the second day of life. As well, the laboratory data were believed to be indicative of sepsis if absolute neutrophil count is <7,800/mm³ or >14,500/mm³ in the first 60 hour, and <1,750/mm³ or >5,400/mm³ after 60 hour of life [16] or serum CRP ≥ 10 mg/l [23-25]. Moreover, bacteriological findings such as positive cultures of blood, urine, cerebrospinal fluid or other body fluids were considered as sepsis bacteriological data. Accordingly, in the presence of ≥3 of abnormal clinical criteria, laboratory data, and bacteriological findings sepsis were considered [23,25,26].

Clinical data collection

All included neonates were subjected to thorough history taking including prenatal, natal as well as postnatal ones. This history was involving maternal infection history during pregnancy (chorioamenionitis, maternal fever), prolonged premature rupture of membranes, mode of delivery, gender of neonates, history of medications and therapeutic interventions. Additionally, all neonates were subjected to careful clinical assessment containing Apgar score estimation at 1 and 5 minutes [27], gestational age, weight at sampling, length, head circumference, vital signs, pallor or jaundice as well as respiratory, cardiovascular, abdominal and full neurological examinations. Total fluid administration and urine output were recorded as well. Chest radiograph had been requested when neonates presented with signs and symptoms suggestive of chest disease. Furthermore, abdominal radiograph has been requested when there were signs and symptoms suggestive of intra-abdominal disease.

Samples collection

Blood samples were collected under complete sterile conditions from all subjects for blood culture, hematological and biochemical laboratory tests. Blood samples from neonates suspected of sepsis were withdrawn at time of sepsis onset before initiation of antibiotic therapy and 72 hours later. Complete blood count (CBC) samples were collected in anti-coagulants EDTA tubes and CBC was performed immediately. For cultures, blood was inoculated into blood culture bottles with specific media. Serum was separated following sample clotting in plain tubes by centrifugation and analyzed immediately for blood urea, serum creatinine, random blood sugar (RBS) and serum C reactive protein. The remaining serum was stored at -70°C for further evaluation of SAA.

Urine samples were collected aseptically in urine bags from suspected sepsis neonates at the onset of sepsis and before initiation of antibiotics plus 72 hours later for measurement of uIL-18. We collected urine samples from control aseptic subjects and from all eligible septic patients. The collected urine was evacuated directly into sterile containers and then aliquoted in empty tubes. The urine samples were then centrifuged at 1500 rpm at 4°C for 10 minutes and the supernatants were separated and stored at -70°C until used for detection of uIL-18 later on.

Microbiological examination

One to two ml of blood was inoculated aseptically into blood culture media after that the bottles were incubated at 37°C for 5-7 days. Positive blood cultures were subsequently sub-cultured on blood agar. The isolated microorganisms were identified by standard bacteriological methods. Blood cultures were positive in 56.7% of patients and they were negative in 43.3% of patients in septic group I. The identified bacteria included *Staphylococcus aureus* (23.5%), *Klebsiella* (35.3%), *Streptococcus pneumoniae* (5.9%), *Escherichia coli* (23.5%), *Pseudomonas aeruginosa* (5.9%) and *Entrobacter* (5.9%).

Laboratory methods

Serum C-reactive protein was assayed by the particle enhanced immunonephelometric method using BN II analyser (Dade Behring Marburg GMBH, Germany). Blood urea, serum creatinine and RBS were analyzed using automated colorimetric method (Mindray BS-800) while CBC was evaluated by automated blood counter (Sysmex KX-21N). Values of WBCs count, hemoglobin (Hb) and platelets count were noted. Peripheral blood smears were stained by Leishman stain. Glomerular filtration rates (GFR) and immature to total neutrophils count (I/T) ratios were calculated for all subjects. These investigations were routinely performed to all neonates [28]. GFR was calculated according to the following equation:

GFR (ml/min/1.73 m^2)=δ × height (cm)/p cr where δ=0.45 if full term and 0.33 if preterm [29].

SAA and uIL-18 were specific markers measured in the serum and urine of all subjects respectively. SAA was assayed by using enzyme immunosorbent assay (EIA) kit from ASSAY PRO, USA, with catalog No. EA8001-1. The assay was performed according to the manufacturer's instructions. Final SAA values were expressed in microgram per milli (μg/ml). As well, IL-18 was measured in urine using a human IL-18 EIA kit (eBioscience BMS267/2, Vienna, Austria). This kit specifically detects the mature form of IL-18 with very low cross-reactivity with pro-IL-18 [12]. The assay was performed according to the manufacturer's instructions. Final uIL-18 values were expressed in picograms per milligram of urinary creatinine (pg/mg ucr).

Statistical analysis

The collected data were statistically analyzed using statistical package for social sciences (SPSS) program version 20.0 (SPSS Inc.,Chicago, IL, USA). Quantitative results were presented as mean ± standard deviation (SD) while qualitative data were presented as per cents (%). Results were expressed as tables and figures. Graphics were done by Excel Microsoft Office 2010. Student t-test was used to compare results between groups as regards quantitative variable and Z-score test was used to compare between two proportions. p-values equal to or less than 0.05 are statistically significant. Correlation was performed by using Pearson correlation coefficient (r). ROC curve was used to evaluate the diagnostic performance of CRP, SAA as well as uIL-18 in neonatal sepsis group versus control group. Moreover, ROC curve was used to evaluate the diagnostic performance of uIL-18, SAA and CRP parallel to each other.

Results

A total of 275 neonates were divided into 125 neonates who were considered infected (septic Group I) and 150 were not infected (control Group II). The septic neonates were further subdivided into two subgroups according to the onset of sepsis. The first subgroup included 58 neonates who were identified as having EOS as diagnosed within the first 72 hours of life and the remaining 67 neonates were identified as having LOS with diagnosis after 72 hours of life. The mean gestational age of septic neonates was 33.08 ± 3.6 weeks; their mean age of sampling was 14.6 ± 9.8 days and their mean weight was 1.7 ± 0.5 kg. In addition, the mean gestational age of control neonates was 38 ± 2.2 weeks; their mean age of sampling was 5.5 ± 3.6 days and their mean weight was 2.7 ± 0.6 kg. Sex of neonates, APGAR score at 5 minutes and mode of delivery were demonstrated as well (Table 1). We further analyzed the clinical characteristics of neonates in the EOS and LOS groups. Comparison of data among these two subgroups is shown in Table 2.

Regarding laboratory data, comparison between septic and non-septic groups is shown in Table 3. There was a highly statistical significant increase in the levels of CRP, uIL-18 and SAA in the septic group (p<0.001). There was also highly statistically significant difference with respect to CBC elements (HB, WBCs count, platelets count and ANC) where p ≤ 0.001. Table 2 shows that there was statistically significant higher level of SAA in LOS group than in EOS group where p=0.04 with no statistically significant difference regarding uIL-18 level.

We were following up our patients clinically and laboratory after 72 hours from the onset of sepsis for the prognostic values of the studied markers. Table 4 shows statistically significant lower levels of uIL-18 and SAA in the second follow up samples which collected 72 hours after the onset of sepsis (p<0.001), with the least level for SAA (110.4 ± 39.5 μg/ml at onset versus 23 ± 36 μg/ml after 72 hours). However, the values of CRP were statistically significantly higher in follow up samples than the onset ones (20 ± 14.8 mg/L at onset versus 61.5 ± 24.6 mg/L after 72 hours) (p<0.001).

		Septic group (Group I) (N=125)	Non-septic group (Group II) (N=150)
Gestational age (week)		33.08 ± 3.6	38 ± 2.2
Preterm neonates		90 (72%)	50 (33.3%)
Sex of neonates (♂/♀)		75/50	100/50
APGAR	**1 min**	6.1 ± 1.58	8.2 ± 0.77
	5 min	8.57 ± 0.63	9.2 ± 0.41
Age of sampling (day)		14.6 ± 9.8	5.5 ± 3.6
Weight at sampling (kg)		1.7 ± 0.5	2.7 ± 0.6
Mode of delivery (NVD/C.S.)		63 (50.4%)	65 (43.3%)

N: Number; NVD: Normal Vaginal Delivery; C.S.: Caesarean Section

Table 1: Comparison between septic and non-septic groups regarding demographic features.

	Septic group (Group I) (N=125)	Non-septic group (Group II) (N=150)	p-values
Urea (mg/dL)	26.7 ± 5.6	24.9 ± 7.05	0.2
Creatinine (mg/dL)	0.3 ± 0.09	0.3 ± 0.1	0.08
GFR (ml/min)	38 ± 14.9	43.9 ± 13.5	0.09
Hb (g/dL)	12.9 ± 3.4	15.6 ± 2.1	<0.001**
Platelets (cells/μL)	89000 ± 80200	257000 ± 62931	<0.001**
WBCs (cells/μL)	19400 ± 18700	10573 ± 3462	0.001*
ANC (cells/μL)	11148.5 ± 7537.7	3876.6 ± 1363.1	<0.001**
CRP titre (mg/L)	20.1 ± 14.8	4.8 ± 3.7	<0.001**
uIL-18 (pg/mg ucr)	73.7 ± 15.7	41.4 ± 6.4	<0.001**
SAA (μg/ml)	110.4 ± 39.5	4.8 ± 1.3	<0.001**

Hb: Hemoglobin; WBCs: White Blood Cells; ANC: Absolute Neutrophils Count; CRP titer: C-Reactive Protein titer; uIL-18: Urinary Interleukin-18; SAA: Serum Amyloid A; GFR: Glomerular Filtration Rate
Non sig.>0.05, Sig.<0.05*, High sig.<0.001**

Table 2: Comparison between septic and non-septic groups regarding laboratory data.

	Group Ia EOS (N=58)	Group Ib LOS (N=67)	p-values
Gestational age (week)	33.8 ± 3.6	32.5 ± 3.5	0.2
Preterm neonates	38 (65.5%)	52 (77.6%)	0.1
Sex of neonates (♂/♀)	38/20	37/30	0.2
Age of sampling (day)	6.7 ± 1.9	21.3 ± 8.8	<0.001**
Weight at sampling (kg)	1.8 ± 0.6	1.6 ± 0.5	0.2
Mode of delivery (NVD/C.S.)	25 (43.1%)	38 (56.7%)	0.1
Urea (mg/dL)	26.3 ± 6.8	27.0 ± 4.4	0.7
Creatinine (mg/dL)	0.3 ± 0.09	0.3 ± 0.1	0.5
GFR (ml/min)	46.2 ± 15.7	31.8 ± 10.3	0.001*
Hb (g/dL)	14.1 ± 3.7	12.1 ± 2.9	0.04*
WBCs (cells/μL)	19123.2 ± 13062.9	19597.3 ± 22666.6	0.9
Platelets (cells/μL)	106869.6 ± 19733.3	73814.8 ± 63383.5	0.1
ANC (cells/μL)	13947.9 ± 7973.2	8763.81 ± 6359.9	0.01*
CRP titre (mg/L)	19.0 ± 15.7	20.9 ± 13.9	0.7
uIL-18 (pg/mg ucr)	73.8 ± 20.0	73.7 ± 11.1	0.4
SAA (μg/ml)	97.0 ± 44.1	121.7 ± 31.2	0.04*

EOS: Early Onset Sepsis; LOS: Late Onset Sepsis; N: Number; NVD: Normal Vaginal Delivery; C.S: Caesarean Section; GFR: Glomerular Filtration Rate; Hb: Hemoglobin; WBCs: White Blood Cells; ANC: Absolute Neutrophils Count; CRP titer: C-Reactive Protein titer; uIL-18: Urinary Interleukin-18; SAA: Serum Amyloid A
Non sig.>0.05, Sig.<0.05*, High sig.<0.001**

Table 3: Comparison between EOS and LOS subgroups as regards demographic and laboratory data.

	Septic Group		
	At onset	**After 72 hours**	**p-values**
WBCs (cells/μL)	19400 ± 18700	10600 ± 7100	**0.002***
Platelets (cells/μL)	89000 ± 80200	247300 ± 150500	**0.005***
ANC (cells/μL)	11148.5 ± 7537.7	5696.6 ± 4307.8	0.270
CRP titre (mg/L)	20.0 ± 14.8	61.5 ± 24.6	**<0.001***
uIL-18 (pg/mg ucr)	73.7 ± 15.7	50.3 ± 6.5	**<0.001***
SAA (μg/ml)	110.4 ± 39.5	23.0 ± 36.0	**<0.001***

WBCs: White Blood Cells; ANC: Absolute Neutrophils Count; CRP titer: C-Reactive Protein titer; uIL-18: Urinary Interleukin-18; SAA: Serum Amyloid A
Non sig.>0.05, Sig.<0.05*, High sig.<0.001**

Table 4: Comparison among septic group as regards laboratory data at onset of sepsis and after 72 hrs.

These three markers showed statistical highly significant negative correlation with gestational age and weight of the neonates (p<0.001) (Table 5). Correlations with some laboratory data are shown in Table 5. We found that both uIL-18 and SAA were correlated with CRP in a significant fair positive manner (p<0.001). uIL-18 and SAA were correlated with each other in a moderate positive and significant way (Table 5). During follow-up of septic neonates, 14.4% of them (18 out of 125) were died. We noted that all the dead septic neonates were having very high levels of both uIL-18 and SAA (data not shown).

To determine cut-off levels that balanced the false-positive and the false-negative rates with the best positive predictive value, ROC analysis was performed for CRP, uIL-18 and SAA. ROC curves of CRP, uIL-18 and SAA for discriminating patients with neonatal sepsis from non-septic subjects were shown in Figure 1A. The AUC value of CRP was 0.866 [95% confidence interval (CI)=0.806-0.925, p=0.000]. Additionally, uIL-18 showed an AUC value of 0.934 (95% CI=0.893-0.975, p=0.000) while SAA showed an AUC value of 0.995 (95% CI=0.989-1.000, p=0.000) (Figure 1B). ROC curves of CRP, uIL-18 and SAA for discriminating patients with EOS from those without sepsis were shown in Figure 2A. The AUC value of CRP was 0.877

	uIL-18		CRP		SAA	
	r	**p**	**r**	**p**	**r**	**p**
Gestational age (week)	-0.45	**<0.001****	-0.56	**<0.001****	-0.57	**<0.001****
Weight (kg)	-0.46	**<0.001****	-0.48	**<0.001****	-0.62	**<0.001****
Hb (g/dL)	-0.43	**0.001***	-0.22	**0.002***	-0.37	**<0.001****
WBCs (cells/μL)	0.30	**0.01***	0.09	**<0.001****	0.29	**<0.001****
Platelets (cells/μL)	-0.62	**<0.001****	-0.48	**<0.001****	-0.69	**<0.001****
ANC (cells/μL)	0.44	**<0.001****	0.46	**<0.001****	0.53	**<0.001****
CRP titre (mg/L)	0.41	**<0.001****			0.49	**<0.001****
SAA (μg/ml)	0.70	**<0.001****				

Hb: Hemoglobin; WBCs: White Blood Cells; ANC: Absolute Neutrophils Count; CRP titer: C-Reactive Protein titer; uIL-18: Urinary Interleukin-18; SAA: Serum Amyloid A
r=0.75-1 (strong correlation), r=0.5-0.74 (moderate correlation), r=0.25-0.49 (fair correlation), r=0.1-0.24 (weak correlation) Non sig.>0.05, Sig.<0.05*, High sig.<0.001**

Table 5: Correlation between uIL-18, CRP, SAA levels and both demographic and laboratory data.

Variable	Cut-Off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Neonatal Sepsis vs. non septic controls					
CRP	5.5 mg/L	84%	80%	77.8%	85.7%
uIL-18	58.0 pg/mg ucr	91.2%	100%	100%	93.2%
SAA	8.85 μg/ml	97.6%	100%	100%	98%
EOS vs. non septic controls					
CRP	5.5 mg/L	87.7%	80%	62.5%	94.5%
uIL-18	58.0 pg/mg ucr	86.0%	100%	100%	94.9%
SAA	8.85 μg/ml	100.0%	100%	100%	100%
LOS vs. non septic controls					
CRP	5.5 mg/L	80.9%	80%	64.7%	90.2%
uIL-18	58.0 pg/mg ucr	95.6%	100%	100%	98.0%
SAA	8.85 μg/ml	95.6%	100%	100%	98.0%

CRP: C-Reactive Protein; uIL-18: Urinary Interleukin-18; SAA: Serum Amyloid A; EOS: Early Onset Sepsis; LOS: Late Onset Sepsis; PPV: Positive Predictive Value; NPV: Negative Predictive Value; vs.: Versus

Table 6: Results of measurement of CRP, uIL-18 and SAA in the diagnosis of neonatal sepsis.

[95% CI=0.818-0.936, p=0.000]. Also, uIL-18 showed an AUC value of 0.866 (95% CI=0.780-0.953, p=0.000) and for SAA, an AUC value of 1.000 (95% CI=1.000-1.000, p=0.000) (Figure 2B). Furthermore, ROC curves of CRP, uIL-18 and SAA for discriminating patients with LOS from those without sepsis were shown in Figure 3A. The AUC value of CRP was 0.866 [95% CI=0.806-0.925, p=0.000]. Also, uIL-18 showed an AUC value of 0.991 (95% CI=0.979-1.000, p=0.000) and for SAA, an AUC value of 0.991 (95% CI=0.980-1.000, p=0.000) (Figure 3B).

These ROC curves indicated that a CRP value of 5.5 mg/L yielded the best sensitivity and specificity for differentiating patients with sepsis from those non infected neonates (Table 6). For uIL-18, this best cut-off value that yielded the maximum sensitivity and specificity was 58 pg/mg ucr and the best cut-off figures for SAA was 8.85 μg/ml (Table 6). Moreover, based on these ROC defined cut-off values, the sensitivities and specificities of CRP, uIL-18 and SAA plus their positive and negative predictive values (PPV & NPV) for the diagnosis of neonatal sepsis were shown in Table 6.

Discussion

Despite the progress in the field of antimicrobial therapy and neonatal life support, neonatal sepsis is still a leading cause of high mortality and morbidity. The early and efficient diagnosis of neonatal

Neonatal Sepsis vs. non septic controls
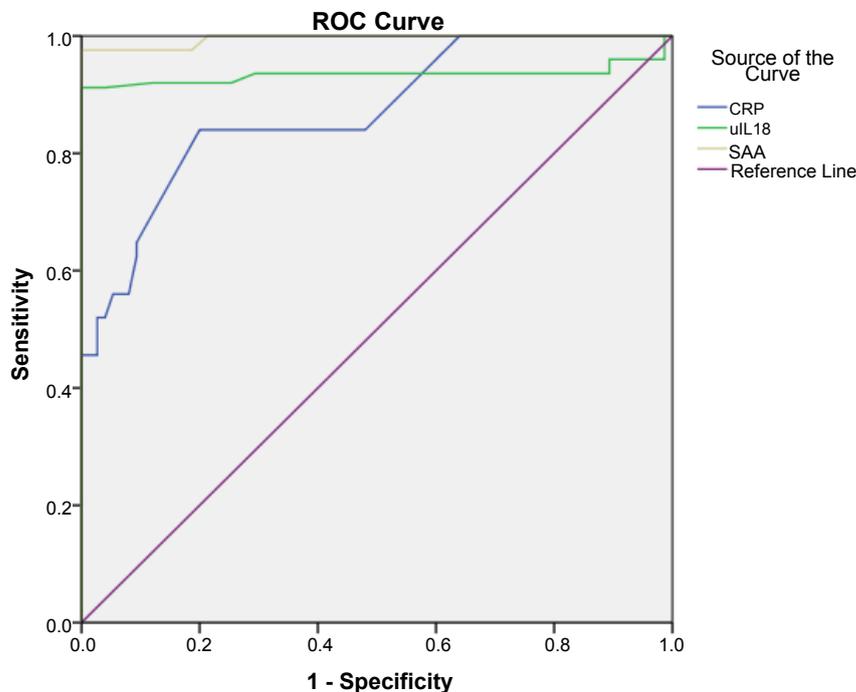
(A)

(B)

Area Under the Curve

Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
CRP	.871	.024	.000	.823	.918
uIL18	.934	.021	.000	.893	.975
SAA	.995	.003	.000	.989	1.000

a. Under the nonparametric assumption
b. Null hypothesis: true area = 0.5

ROC, receiver operating characteristic ; CRP, C- reactive protein; uIL-18, urinary interleukin-18; SAA, Serum Amyloid A

Figure 1: Diagnostic performances of uIL-18 and SAA against CRP for discriminating septic from non-septic neonates. **(A)** ROC curve obtained by plot at different cut-offs for CRP, uIL-18 and SAA in septic group versus non septic controls. **(B)** The area under the curve is 0.871 for CRP with Std. Error=0.024 and 95% Confidence Interval (CI) from 0.823 to 0.918. The area under the curve is 0.934 for uIL-18 with Std. Error=0.021 and 95% CI from 0.893 to 0.975 and the area under the curve is 0.995 for SAA with Std. Error=0.003 and 95% CI from 0.989 to 1.000.

sepsis remains a difficult task, as the clinical signs are insufficient, vague and non-specific. If treatment is delayed till symptoms and signs of sepsis become obvious, the sepsis will deteriorate and may progress to disseminated intravascular coagulation [30,31] and therefore risk of preventable mortality would be brought up [32]. In addition, non-septic neonates such as those with transient tachypnea, meconium aspiration syndrome, respiratory distress syndrome, apnea of prematurity and acute exacerbation of chronic lung disease are often clinically indistinguishable from early sepsis in neonates [33]. Accordingly, reliable infection biomarkers are vital to achieve precise and early diagnosis in neonates while blood culture results are pending. Collectively, early detection of neonatal sepsis is critical to diminish the abuse of antibiotics in NICU, minimize the development of antibiotic resistance and hence reduce the duration of hospitalization. However,

to date, no single sepsis marker is dependable for early identification of infected neonates [34]. This clinical study was performed to evaluate the diagnostic performance of both SAA and uIL-18 in the diagnosis and follow up for neonatal sepsis and to put them side by side with the more widely used marker CRP.

The incidence of sepsis increases in an inverse correlation with gestational age and can be detected as high as 30% in low birth weight infants. The mean gestational age of septic neonates who were involved in this study was 33.08 ± 3.6 weeks; their mean age of sampling was 14.6 ± 9.8 days and their mean weight was 1.7 ± 0.5 kg. The gestational age and weight at the time of sampling were lower in infected neonates than in those without infection. Further, the current study found that 72% of the septic neonates were preterm. These findings were the same as to what reported in Li et al., Shaha et al., Vergnano et al., in addition to Adams-

EOS vs. non septic controls
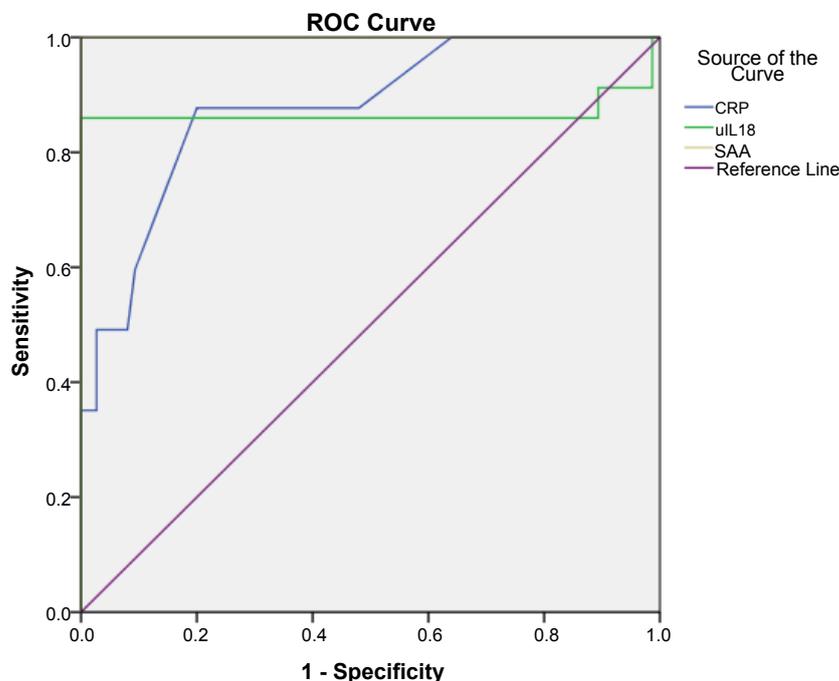
(A)

(B)

Area Under the Curve

Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
CRP	.877	.030	.000	.818	.936
uIL18	.866	.044	.000	.780	.953
SAA	1.000	.000	.000	1.000	1.000

a. Under the nonparametric assumption
b. Null hypothesis: true area = 0.5

EOS, early onset sepsis; ROC, receiver operating characteristic ; CRP, C- reactive protein; uIL-18, urinary interleukin-18; SAA, Serum Amyloid A

Figure 2: Diagnostic performances of uIL-18 and SAA against CRP for discriminating early onset septic neonates from control group. **(A)** ROC curve obtained by plot at different cut-offs for CRP, uIL-18 and SAA in EOS subgroup versus non-septic controls. **(B)** The area under the curve is 0.877 for CRP with Std. Error=0.030 and 95% Confidence Interval (CI) from 0.818 to 0.936. The area under the curve is 0.866 for uIL-18 with Std. Error=0.044 and 95% CI from 0.780 to 0.953 and the area under the curve is 1.000 for SAA with Std. Error=0.000 and 95% CI from 1.000 to 1.000.

Chapman and Stoll who reported that prematurity was one of the risk factors for neonatal sepsis [6,12,35,36]. The underdeveloped immune system predisposes preterm new-borns to infection [37] due to low levels of IgG [38]. Also their low birth weight is a risk factor for neonatal sepsis [39]. Additionally, seeking for improvement in the survival of preterm neonates make them remain in the hospital for a longer time in an environment that puts them at continuous risk for acquired infections [40]. Also, in the current study, there is male predominance (60%). This agrees with the study of Baltimore, who reported that male gender was associated with a higher rate of sepsis [41]. Edwards, correlated this to X-linked immune-regulatory genes [42]. Total WBC and differential counts; an immature-to-total neutrophil ratio ≥ 0.2; neutropenia; thrombocytopenia; plus levels of many sepsis biomarkers in addition to bacterial cultures are all considered as diagnostic panels for neonatal sepsis. Positive blood cultures is the most reliable test that

provide a definitive diagnosis for sepsis however the increase in its false negative and false positive results plus the delayed lag time needed to have an approved data add a great limitations to this laboratory test. Regarding blood cultures in our study, only 56.7% of septic neonates had positive blood cultures. This agrees with Kayange et al., who found that 47% of septic neonates had positive blood culture but disagrees with Mohsen et al., who found that 100% of septic neonates had positive blood culture [43,44]. The difference between studies could be attributed to the diversities in culture sensitivity techniques between labs that can recognize microorganisms as early as possible and thus lead to culture positivity. *Klebseilla* was the dominant organism isolated from the blood of infected group (35.3%) followed by *Staphylococcus aureus* and *E. coli* (23.5%) then by *Pseudomonas*, *Enterobacter*, and *Streptococcus pneumoniae* (5.9%). These results are in agreement with other several studies done by Fathy et al., Abou Hussein et al., and

LOS vs. non septic controls
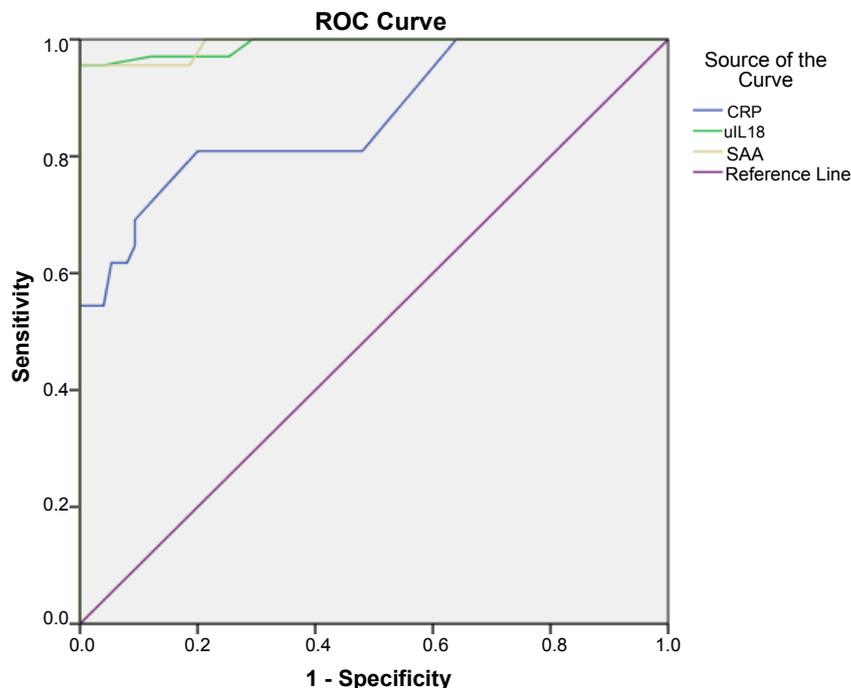
(A)

(B)

Area Under the Curve

Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
CRP	.866	.030	.000	.806	.925
uIL18	.991	.006	.000	.979	1.000
SAA	.991	.005	.000	.980	1.000

a. Under the nonparametric assumption
b. Null hypothesis: true area = 0.5

LOS, late onset sepsis; ROC, receiver operating characteristic ; CRP, C- reactive protein; uIL-18, urinary interleukin-18; SAA, Serum Amyloid A

Figure 3: Diagnostic performances of uIL-18 and SAA against CRP for discriminating late onset septic neonates from control subjects. **(A)** ROC curve obtained by plot at different cut-offs for CRP, uIL-18 and SAA in LOS subgroup versus non septic controls. **(B)** The area under the curve is 0.866 for CRP with Std. Error=0.030 and 95% Confidence Interval (CI) from 0.806 to 0.925. The area under the curve is 0.991 for uIL-18 with Std. Error=0.006 and 95% CI from 0.979 to 1.000 and the area under the curve is 0.991 for SAA with Std. Error=0.005 and 95% CI from 0.980 to 1.000.

Hashim et al., who reported that *Klebseilla* is the commonest isolated organism in septic new-borns with a ratio from 35-56% [45-47].

There are varies diagnostic markers of neonatal sepsis either established or under investigations. These markers include CRP, procalcitonin, haptoglobin, fibrinogen, and cytokines (interleukin (IL) 6, IL-8, and tumor necrosis factor-α), etc. Among these markers, CRP is most frequently used globally in all hospitals for diagnosis and during follow-up. CRP is a globulin that is produced by hepatocytes in response to tissue injury, trauma, cellular degeneration and infection. CRP levels were reported to be normal at the onset and 6 hours after infection by invasive bacteria then becomes most apparent within 12 to 24 hours with peaks within 2 to 3 days and remains elevated till infection is resolved [48-50]. Additionally, it has been reported that elevation of

CRP levels may not happen until 8 to 48 hours after the first clinical suspicion of infection [51,52]. In the present study, the levels of CRP were statistically significantly higher in neonates with infection. This is in agreement with Harris and Munson, who considered CRP as one of the sepsis screening parameters [39]. CRP was positive (>6 mg/L) in 63.3% of septic neonates at onset of sepsis and in 100% of septic neonates after 72 hours. Also, this is in accordance with Awad et al., who found positive CRP in 56.7% of septic neonates in early readings and became 100% after 48 hours [53]. They explored that serial CRP measurements were shown to be more accurate than a single one when evaluating neonates with suspected infection [54]. Additionally, Shortland et al., reported that CRP remained normal in 54% of very low-weight premature infants during culture positive neonatal sepsis [55]. Therefore, finding more reliable diagnostic as well as prognostic

markers for neonatal sepsis with high sensitivities as well as specificities is fundamental.

SAA has been reported to have an anti-inflammatory effect through many ways including oxidative respiration of neutrophils, neutralizing the pyrogenic consequences of various cytokines, minimizing the production of prostaglandin E2, inhibiting platelet activation, controlling the production of antibodies and inducing the secretion of collagenase by fibroblasts. Hence, it has been suggested as a sepsis biomarker [52]. Our data demonstrated that SAA was statistically significantly higher in septic neonates than in non-septic (p<0.001) and there was statistically significant difference in the level of SAA between EOS and LOS groups in favor to LOS (p=0.02). This is in agreement with Arnon et al., who found that SAA value was significantly higher in septic in comparison to non-septic neonates [56,57]. Arnon et al., reported that SAA had an overall better diagnostic accuracy for early prediction of LOS in preterm neonates than CRP [58]. Moreover, we have also shown that it turned down faster than CRP during the follow-up of sepsis after 72 hours. This is in agreement with Cetinkaya et al., Arnon et al., and Arnon et al., as well as lots of other literatures who found that medians of SAA were high at onset of sepsis compared to 48 or 72 hours after its onset. They reported an earlier and sharper manner of SAA elevation than CRP [16,17,56,58].

Additionally, several studies have tried to find reliable early reacting cytokines for detection of neonatal sepsis [59]. Because of variable degrees of non-specific inflammation during the first three days of life [60], evaluation of possible infection is sometimes extremely difficult especially in the EOS. Given their non-invasive collection and ease of accessibility, development of biomarkers for neonatal sepsis has recently focused on the use of saliva and urine as surrogates for early infection detection or risk [61]. Suguna et al., tested the usefulness of urinary cytokines IL8, IP10 and MCP-1 in evaluation of neonatal sepsis and they have been shown to be elevated during early presumed infection in a cohort of healthy and at-risk term infants, identifying a role for urine as a potential biomarker habitat in infant early infection [62]. Similarly, the concentrations of uIL-18 were significantly higher in the presence of sepsis [63,64]. In this study, uIL-18 level was statistically significantly higher in septic than in non-septic neonates. This agrees with Li et al., who found that uIL-18 similar to CRP is a specific marker for predicting the presence of sepsis in critically ill neonates but in the study by Li and coauthors the sample size of infected neonates was small [12]. Unlike SAA, there was no statistically significant difference between EOS and LOS regarding uIL-18 levels but there was statistically highly significant difference between uIL-18 level at onset of sepsis and after 72 hours. The mean value at onset was 73.7 ± 15.7 pg/mg ucr while that after 72 hours was 50.3 ± 6.5 pg/mg ucr (p<0.001). Kingsmore et al., found that IL-18 levels were highly associated with sepsis [65]. Likewise, Harris et al., showed that IL-18 levels increased in septic neonates versus neonates with necrotizing enterocolitis [66].

Altogether, our results show that both SAA and uIL-18 rose in a sharper manner at the onset of sepsis then decreased faster compared with CRP in neonates who started to recover after 72 hours from the onset of sepsis as demonstrated by the improvement in the clinical as well as the laboratory data. This is in agreement with Arnon et al., and Cetinkaya et al., [16,58] regarding SAA in parallel with CRP. On the other hand, CRP levels remained elevated after these 72 hours and so not matched with other laboratory findings along with clinical features. Collectively, these finding are in concurrent with those demonstrated by Cetinkaya et al., where the follow up period was 48 hours [16]. Additionally, the levels of SAA and uIL-18 in 18 septic neonates (14.4%)

-who deteriorated and died later on- were initially higher than their levels in survived neonates and these levels were remained unchanged after 72 hours. Accordingly, SAA and uIL-18 may have better early diagnostic and prognostic values than CRP. However, the prediction of mortality was not taken into consideration statistically; because of the low number of died cases and the shortness of the duration of the follow-up. The prediction of mortality should be considered later on in prospective studies to shed more light on the accuracies of the SAA as well as uIL-18 as prognostic biomarkers for neonatal sepsis.

According to what stated above, it is obvious that CRP is not efficient enough as neonatal sepsis biomarker. Moreover, CRP had low specificity as it showed an elevation in a number of infectious and non-infectious diseases (inflammation or tissue injury). Normal CRP values during the first 24 to 48 hours of sepsis cause a 99% negative predicted value. In contrast, elevated levels of CRP may be more difficult to interpret, especially for diagnosis of EOS because of PROM, maternal fever, pregnancy-induced hypertension, prenatal steroid use, and fetal distress [67]. Additionally, studies have suggested a physiologic variation of the CRP during the first few days of life. Gestational age as well influences CRP kinetics, with preterm infants having a lower and shorter CRP response compared to healthy term infants [67]. Consequently, CRP is best used in combination with other biomarkers with better specificities plus high sensitivity rather than as a single test. For that to be achieved, numerous head to head studies with large sample sizes should be conducted. These studies have to be carried out on different populations as well as gestational ages because it has been stated that the incidence of neonatal sepsis varies according to age, weight and race [67]. For example, different clinical studies for sepsis burdened by gestational age and race have shown that black preterm neonates have a significantly higher incidence of neonatal sepsis as compared to other population, accounting for 5.14 cases/1000 births with a case fatality rate of 24.4% [68]. In this study, we planned to establish whether SAA or uIL-18 could improve diagnosis in this most challenging patient group in a parallel head to head comparison with CRP. We employed the ROC curve to estimate the diagnostic usefulness of these markers in a multicentre recruitment case-control study among Egyptian neonates.

Based on our ROC curve analysis, the AUC for distinguishing neonatal sepsis as a whole from control group was 0.871 regarding CRP along with 0.934 & 0.995 for uIL-18 and SAA respectively. As a result, both markers showed more usefulness than CRP. Thus, it is obvious that SAA has the highest diagnostic efficacy for neonatal sepsis, whereas uIL-18 is moderately efficient and the least for CRP. Our results suggest that the best cutoff value for diagnosing neonatal bacterial infection is 58 pg/mg ucr for uIL-18, 8.85 μg/ml for SAA and 5.5 mg/L for CRP. These cut-off points are the ones that yield the best sensitivities and specificities. uIL-18 had a sensitivity of 91.2% and specificity of 100% and SAA had a sensitivity of 97.6% and specificity of 100% as well while CRP had a sensitivity of 84% and specificity of 80%. It is clear that SAA showed the best performance followed by uIL-18 then CRP with the least diagnostic outcomes. Additionally, the sensitivity and specificity were the lowest for CRP in comparison to the other two markers. Thus, the combination between these markers and CRP could be beneficial in elevating the diagnostic performance of the traditional marker, CRP. Furthermore, the performances of all three markers were analyzed but concerning either EOS or LOS against control neonates. Again, SAA had the most upper hands over the other 2 markers for distinguishing EOS while CRP performance slightly gets over that of uIL-18. As Regards LOS, the performance of uIL-18 had risen up to be the same as SAA which are both elevated very tremendous than that of CRP.

The actual impact of diagnostics depends on the availability and performance of the test, as well as the effectiveness of the treatment based on the test results. Previous studies have reported the sensitivity and specificity of either CRP or SAA but very few studies detected that for uIL-18 [12,17]. The performance of SAA had been extensively investigated in lots of previous studies. SAA diagnostic efficacies were very well in four studies done by three different groups (sensitivity 96%-100%, and ROC AUC of 0.94-0.997) [57,58,69,70], and performed moderately in another study, with a sensitivity of 76% and a ROC AUC of 0.875 [58]. In contrast Edgar et al., showed poor performance of SAA with 24% sensitivity and 0.61 ROC AUC, although the specificity was 93% [71]. The cut-offs used in these studies varied considerably, from 0.8 mg/L to 1000 mg/L. However, the three studies that used a cut-off of 50 mg/L or less showed good sensitivity. Cetinkaya et al., and Enguix et al., reported that SAA and CRP had the same diagnostic efficiency and the same area under the ROC curve in neonates with bacterial sepsis [16,70]. Similar to our study, Arnon et al., found that SAA had significantly largest AUC compared with CRP at 0 h of sepsis evaluation [56]. Heterogeneity between studies could be explained by the use of diverse cut-off point, dissimilar SAA assays (EIA, Latex, Automated immunoassay and Immune-nephlometric assay) beside different ages of included neonates [17]. This data suggests that SAA could be meaningful and robust biomarker in the diagnosis of serious new-born infections but establishing an appropriate cut-off concentration for its diagnostic performance is yet critical.

To the best of our knowledge, the diagnostic capabilities of uIL-18 have not previously been studied in comparison to SAA among infected neonates. Moreover, its performance on neonatal sepsis had been very rarely investigated either alone, in comparison with CRP and/or any other infection markers. Li et al., had studied uIL-18 performance in critically ill children but as mentioned above, sample size of infected neonates was small (26 out of 120) [12]. As well, Lauw et al., found that plasma IL-18 was raised but in infected adults [72]. Also, Cui et al., stated that both plasma and miRNA expression levels of IL-18 increased in severe septic adults in correlation with thrombocytopenia [73]. Likewise, the current study revealed a significant moderate and negative correlation between uIL-18 and platelets count in neonatal sepsis.

There are many advantages of our real-life assessment study which give the study a clinical significance. These advantages include the bigger number of the control subjects than patients. Another advantage is that the investigated patients were from multicentre recruitment because these subjects were from two centers located in two regions within Egypt and this should act as an external validation to this study. Also, we compared the performance of SAA and uIL-18 to CRP which is the most widely used sepsis biomarker. However, there are important limitations to the current literature. The limitations involve: 1) the short time of follow up and the lack of measuring the dynamic changing patterns in the levels of these three markers within shorter time frequencies. 2) The relatively small numbers of participants which are due to financial issues. 3) The increased number of premature or low birth-weight infants which adds a significant heterogeneity to the neonates involved in this study. This is attributed to both the nature of the study itself which included almost all septic neonates in certain areas with an unselected manner and within exact time limit in addition to the fact that implies premature neonates as the most vulnerable population to infection [36]. Larger validation studies as well as more detailed follow up ones focusing on combinations of these promising biomarkers are necessary in order to determine their true performance characteristics. Additionally, comparison between uIL-18 and serum/plasma IL-18 could be valuable in prospective studies. This could help in predicting the development of sepsis or the progression of its severity and consequently assist in reducing global infant mortality.

Conclusion

Collectively, our results indicate that both SAA and uIL-18 could compete or synergize with CRP as markers for sepsis screening in critically ill neonates. Measurement of SAA and uIL-18 in suspected cases may increase the accuracy of diagnosis particularly because uIL-18 has the advantage of being non-invasive biomarker. Moreover, uIL-18 and SAA may have prognostic value in the follow up of septic neonates which merits further investigation.

Ethical Considerations

The study was operated in accordance with the World Medical Association's Declaration of Helsinki. This study was approved by the research ethics committee of El-Minia University. The purpose, nature and potential risks of the experiments were fully explained to the parents. All parents gave written, informed consents at the beginning of the study and all data were kept confidential and used for research purposes only.

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