Combination of Procalcitonin, CRP and CD11b Biomarkers in Early Detection of Neonatal Sepsis

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The study aimed at comparing the diagnostic performances of CRP, PCT and CD11b in neonatal sepsis and evaluating the effectiveness of the sepsis score system when using a combination of various biomarkers. The study was conducted on 90 neonates divided into 3 equal groups; a group with proven sepsis, suspected sepsis and healthy newborns. All were subjected to measurement of CPR by Latex agglutination, serum Procalcitonin by ELISA and CD11b by flow cytometry. On comparing the three biomarkers; PCT (Serum procalcitonin) was associated with the highest (AUC) area under the curve followed by CD11b and CRP recording the smallest value. However, the AUC of the combined sepsis score was much higher than individual biomarkers. Although the sensitivity of individual biomarkers from procalcitonin to CD11b and lastly CRP but the sensitivity and specificity of the sepsis score showed higher values compared to those of individual biomarkers. In conclusion, the study demonstrate that combination of CRP, CD11b and, procalcitonin can enhance diagnostic discriminative power over traditional tests and overcome the drawbacks of each test alone with greater diagnostic accuracy.

Despite the immense advances in neonatal intensive care, neonatal sepsis remains a leading cause of neonatal morbidity and mortality [1]. In 2017, it caused death of around 5.4 million children under the age of 5 with 2.5 million of them occurring during the first month of life. The majority of these deaths usually occur in low-income countries [2].

Early diagnosis and treatment of suspected cases of neonatal sepsis are both essential to prevent life-threatening complications but still offer a major challenge because clinical signs are non-specific, variable and resemble those caused by various non-infective conditions [3].

Although a large number of biomarkers were introduced for the diagnosis of sepsis, none of them has shown conclusive results in clinical settings. Thus, in a search for a solution combination approaches measuring multiple biomarkers have been proposed [4].

CD11b is a cell surface antigen, also known as Integrin alpha M (ITGAM) or complement receptor 3 A (CR3A) is normally expressed on the surface of neutrophils at very low levels in non-activated cells and its expression level increases within few minutes after cell exposure to bacterial infection or endotoxins [5]. CD11b mediates inflammation by regulating leukocyte adhesion and migration. In addition it is involved in phagocytosis, cell-mediated cytotoxicity, chemotaxis, and the complement system. CD11b was reported to be a highly effective marker in the diagnosis of early-onset neonatal infection with elevated expression when compared to non-infected neonates [6].

Serum procalcitonin (PCT), is a prohormone of calcitonin that is produced by the C-cells of the thyroid gland and its circulating levels are undetectable but

increase exclusively in response to bacterial infection only and not to other viral or inflammatory diseases [7]. This allows PCT to be a promising candidate marker for identification of bacterial sepsis [8]. On the other hand, serum levels of PCT increase briskly within 2–6 h after the exposure making it a rapid diagnostic marker compared to culture methods [9]. PCT values can be used to help doctors in taking decisions on when to start and stop antibiotic therapy for bacterial infections [10].

In the current study we aimed to compare between the accuracies of CRP, PCT and CD11b as diagnostic markers for neonatal sepsis and evaluate the effectiveness of the sepsis score system which depends on the combination of various biomarkers and to assess the validity of the combination approach.

Materials and Methods

Settings

The study was conducted in the Neonatal Intensive Care Unit (NICU) and Microbiology department at Benha university hospital, the study was approved by the Research Ethics Committee, Faculty of Medicine, Benha University, Egypt. Consents were provided by the neonate's parents for participation in this study.

Study design

This case-control study was conducted on ninety neonates in the first 28 days of life who were admitted to the Neonatal Intensive Care Unit (NICU) at Banha university hospital during the period from January 2018 to October 2018. The neonates were divided into 3 groups each including 30 neonates; a proven sepsis group with clinical signs and symptoms of sepsis and positive blood cultures, a suspected group with signs and symptoms of sepsis but negative blood cultures and a control group of apparently healthy newborns of comparable age and sex taken randomly from the follow up clinic.

Exclusion criteria included congenital abnormalities, congenital infections, hypoxic ischemic encephalopathy, birth injuries & metabolic disorders.

Sample Collection and Biomarker Assays

5 ml of venous blood was collected by sterile venipuncture. Two ml of blood were mixed with blood culture medium, the remaining part was divided into two parts: EDTA free part for ELISA, the other part was collected in EDTA containing blood collection tube and processed as whole blood within 24h after venipuncture for flow cytometry.

All the study population was subjected to the following: history taking, clinical examination and laboratory investigations, including: complete blood count by Sysmex automated cell counter, Blood cultures by the BACTEC microbial detection system (Becton – Dickinson sparks, MD), measurement of CRP uses Latex agglutination test, Rapitex CRP kit. It was considered positive when the titer was>6mg/L by Horiba ABX micro CRP 200(TS85213a9)

- Measurement of serum Procalcitonin

Human procalcitonin was measured by Human Procalcitonin DuoSet®ELISA kit, Catalog Number: DY8350-05 (R&D Systems, Inc. USA). The detection limits ranged from 27.43-20,000pg/ml. ELISA procedures were done according to the instructions of the manufacturers. The optical density of each well was determined immediately, using a microplate reader set to 450 nm. Obtained optical density values, were converted into pg/mL by the Bio Rad ELISA data analysis software. All experiments were performed in duplicate and data represents mean values. The cut-off value was calculated as the mean absorbance value of the negative controls plus three standard deviations.

- Assay for CD11b by flow cytometry

100 µl of the sample was added to 10 µl Fluorescein phycoerythrin, (PE) anti-CD11b mouse monoclonal antibody (BD Pharmingen (San Diego, CA, USA), vortexed, and incubated at 4°C for 20 min in the dark. RBCs were lysed using FACS Lyse solution (NH_4Cl buffered with HCO_3 at pH 7.2). (Becton Dickinson), In brief, one and half milliliters of the Lysis solution was added to each tube, vortexed, and incubated for 15 min at room temperature. Tubes were centrifuged at 2000 rpm for 5 minutes, and the supernatant was discarded. Cells were washed twice with 2 ml PBS, with repeating centrifugation, followed by discarding of the supernatant. An isotype matched negative control sample was used. FITIC Mouse IgG1 (Catalog Number: IC002P, R&D system, USA) and PE Mouse IgG2a (Catalog Number: IC003P, R&D system, USA) isotypes were used as negative control sample in all cases to assess background fluorescence

intensity. (Fig 1-b). Labelled cells were then ready for processing on the flow cytometer. Data were acquired on a FACS caliberflow cytometer (Becton Dickinson immune cytometry systems, San Jose, CA, USA). The Data were analyzed using the CellQuest software program (BD Biosciences). Neutrophils were electronically selected on the basis of their side –and forward –scatter characteristics (Fig 1-a). Ten thousand events were measured for each sample. The percentage of CD11b expressing cells and the level of expression per cell was calculated: delta (Δ) MFI (mean fluorescence intensity) = MFICD11b-MFI isotype. (Fig.1-c, d, e).

Statistical Analysis

Data were tabulated, coded and analyzed using the Statistical Package for the Social Sciences (SPSS) software version 23.0 for Windows. Qualitative date was expressed in number and percentage while quantitative data was expressed in mean and standard deviation. For comparisons of more than two groups, ANOVA was used for normally distributed data, and the Kruskal–Wallis test for non- normally distributed data. The diagnostic test efficiency was evaluated by receiver operating characteristic (ROC) curve analysis. The area under curve (AUC) was calculated for different ROC curves of single biomarkers and for the combined sepsis score. AUC is an indication of the diagnostic performance of diagnostic tests. Optimal cut-off values of PCT, CD11b and CRP and different combination of them were calculated based on the combination of sensitivity and specificity. A *P* value of <0.05 was considered significant. A sepsis score was calculated by combining the values of CRP, CD11b and PCT into a single value using logistic regression models as was previously done by Thakur *et al.*, 2017 [11]. The sepsis score is the value of the predicted probability of sepsis calculated based on logistic regression analysis. The calculations method is summarized as follow: Step 1: Logistic Regression analysis was performed using CRP, procalcitonin and CD11b levels as predictor variables, and the presence or absence of sepsis as the dichotomous dependent variable to determine the logistic regression coefficients β_0, β_1. β_n. Step 2: Calculate Logit: $(L) = \beta_0 + \beta_1 X_1 + + \beta_n X_n$ Where β_0, β_1. β_n are the logistic regression coefficients and X_1, X_2... X_n are the independent predictor variables. Step 3: Calculate estimated probability: $(\hat{P}) = (e^L)/(1+e^L)$ Where L is the Logit calculated in step 1. Step 4: Sepsis score = $\hat{P} \times 100$

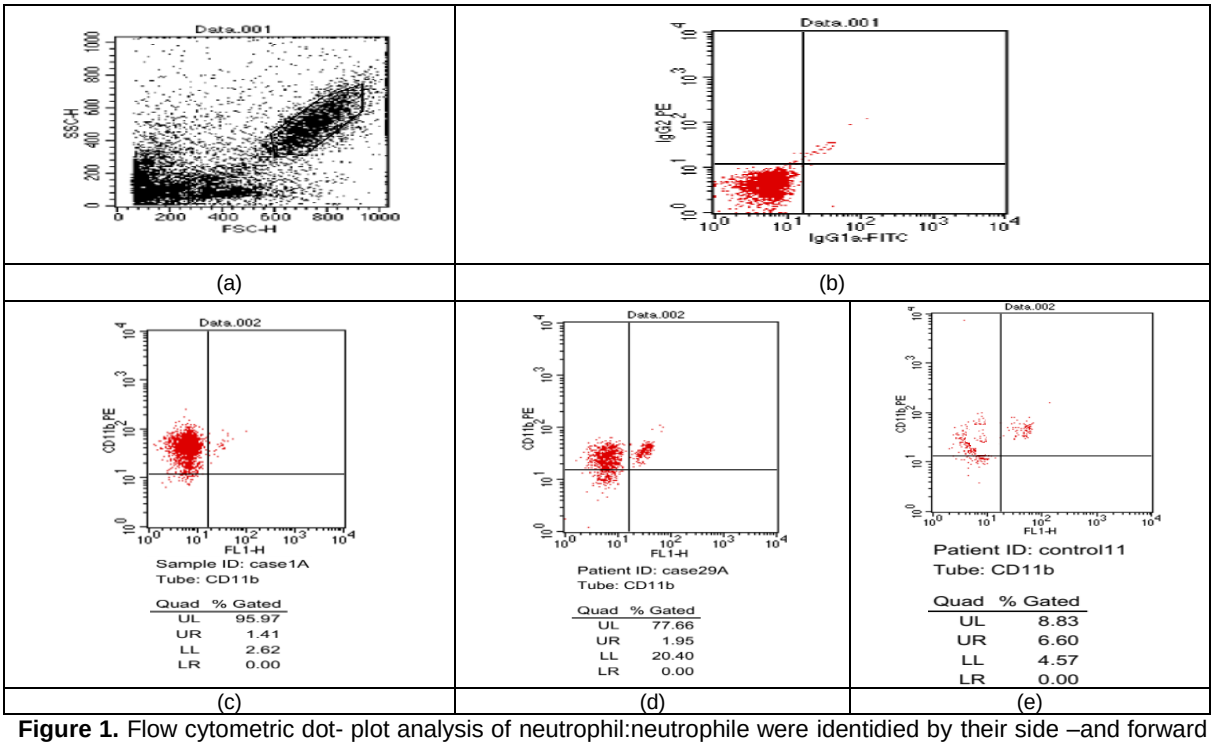

Figure 1. Flow cytometric dot- plot analysis of neutrophil:neutrophile were identidied by their side –and forward –scatter characteristics (fig 1-a),An isotpes negative control (fig 1-b).CD11b expression :a Proven sepsis(fig1-c),a clinical sepsis (fig 1-d)and healthy control (fig1-e) respectively.

Results

This study was conducted on ninety neonates who were divided into 3 equal groups: Proven sepsis, Clinical Sepsis and Control group. Data of cases and control were summarized in Table 1.

Results of blood cultures taken from septic neonates showed that the Gram-negative Klebsiella has been the most frequently isolated organism (40%) followed by *Staph aureus* (36.6%), *E. coli* (10%), Candidiasis (6.6%), Pseudomonas (3.3%) and Serratia (3.3%).

CRP, PCT and CD11b expressions were significantly elevated (P<0.001) in infected neonates as compared with control group. CD11b expression levels, plasma PCT concentrations, and CRP concentrations all increased in the following order: proven sepsis group> clinical sepsis group> healthy control group (table 2).

The ROC curve analysis was used to determine the diagnostic performance of each biomarker and the calculated sepsis score (Fig 2).

Table 1. The demographic characteristics of the three study groups

	Proven sepsis Group Ia	Clinical Sepsis Group 1b	Control group Group II
Number of neonates	30	30	30
Male/female	21/9	15/15	14/16
Gestational age (wk) [Mean ± SD (range)]	35.7±2.35 (32-42)	35.1±2.68 (30-42)	37.6±1.07 (36-40)
Birth weight (kg) [Mean ± SD (range)]	2.6±0.42 (1.86-3.725)	2.6±0.56 (1.4-4)	3.2±0.38 (2.5-4)
Postnatal age (d) [Mean ± SD (range)]	9.2±5.17 (2-22)	11.6±7.11 (1-27)	7.4±4.56 (1-20)

Table 2. C-reactive protein, PCT and CD11b expressions among different groups

		Proven sepsis Group Ia Mean ± SD	Clinical Sepsis Group Ib Mean ± SD	Control Group II Mean ± SD	*P*-Value
Single Test	PCT	5.87±8.97	3.00±5.43	0.51±0.47	<0.001
	CRP	71.63±53.71	19.6±21.51	4.0±2.15	<0.001
	CD11b	284.31±66.36	144.52±31.81	112.18±13.82	<0.001
Combined Sepsis score		228.52±194.68	91.85±119.31	16.68±9.87	<0.001

P<0.05 is significant

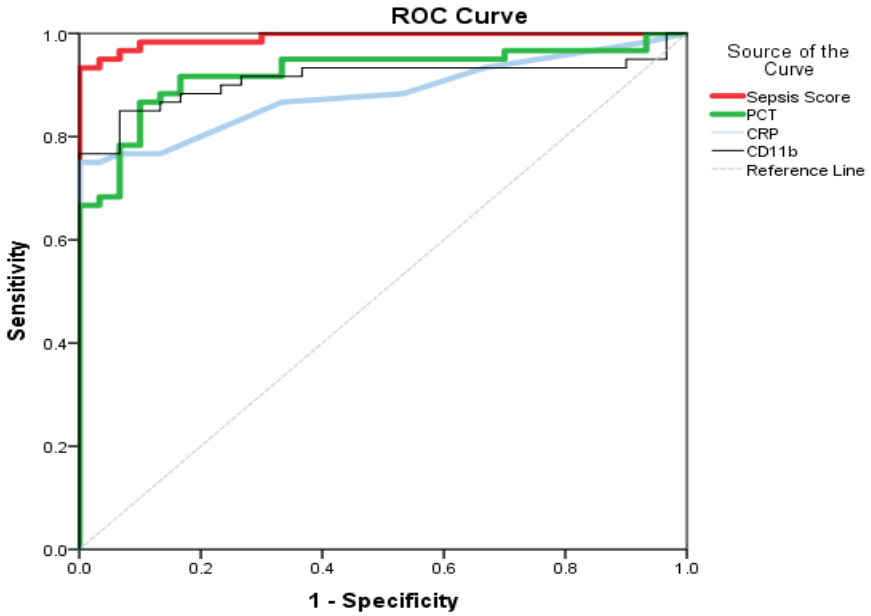

Figure 2. Receiver-operating characteristic curves of PCT, CD11b, CRP, and sepsis score in differentiating between the presence and absence of sepsis. Areas under the receiver-operating characteristic curves for CRP (0.883 [95% CI,. 814–0.951]); PCT (0.923 [95% CI, 0.866–0.980]); CD11b (0.912 [95% CI, 0.848–0.975]); and Sepsis score ([95% CI, 0.848–0.975]). CI = confidence interval; PCT = procalcitonin.

The area under curve (AUC) was calculated for different biomarkers and for the sepsis score (Table 3). When testing a single biomarker the highest AUC was achieved by PCT (0.92 [95 CI: 0.87-0.98; *P*<0.001]) followed by CD11b (0.91 [95 CI: 0.85-0.98; *P*<0.001]) and lastly CRP (0.88 [95 CI: 0.81-0.95; *P*<0.001]). But the AUC of the combined sepsis score was much higher compared to the AUC of any single biomarker [0.99 [95 CI: 0.98-1; *P*<0.001].

Table 3. Area under curve (AUC) for measured biomarkers in diagnosis of sepsis.

Biomarker	AUC	
	Area (95% CI)	*P* value
CRP	0.88 (95 CI:0.81-0.95)	<.001
CD11b	0.91 (95 CI:0.85-0.98)	<.001
PCT	0.92 (95 CI:0.87-0.98)	<.001
Sepsis score	0.99 (95 CI:0.98-1)	<.001

P<0.05 is significant

ROC curve analysis was used to determine the optimal cut-off points for diagnostic tests, using the shortest distance to the upper left corner method. Sensitivity and specificity were calculated at the selected cut-off points.

For a single biomarker the greatest sensitivity was achieved by PCT (86.7 (95% CI: 78.1-95.3)), followed by CD11b (85.0 (95% CI: 76.0-94.0)), and lastly CRP (76.7 (95% CI: 66.0-87.4). Sepsis score achieved high sensitivity (95.0 [95% CI: 89.5-100])

and specificity (96.8[95% CI: 90.6-100]). The sensitivity and specificity of sepsis score were higher than all sensitivities or specificities achieved by any single biomarker alone in the study, (Table 4).

Table 4. Cutoff points, sensitivity and specificity of markers

Biomarker	Cut-off*	Sensitivity§	Specificity §	PPV	NPV
CRP	8	76.7 (66-87.4)	93.3 (84.4-100)	96%	67%
CD11b (RFU)	131	85.0 (76-94)	93.3 (84.4-100)	96%	76%
PCT	1	86.7 (78.1-95.3)	90.0 (79.3-100)	95%	77%
Sepsis score	67	95.0 (89.5-100)	96.8 (90.6-100)	98%	91%

NPV = negative predictive value; PPV = positive predictive value; PCT = procalcitonin.

* Cut-offs were determined using the minimum distance to upper-left corner in the ROC curve.

§ Presented with 95% confidence intervals.

Logistic regression analysis (Table 5) revealed that the determinations of PCT (*P* = 0.006), CD11b (*P*= 0.005) and CRP (*P*= 0.047) all contribute to the prediction of sepsis and their contributions are statistically significant.

As a result of logistic regression analysis, the probability of neonatal sepsis can be calculated by the following equation, in which -16.746 is the constant calculated from the regression equation and 3.048, 0.091, and 0.381 are the regression coefficients for PCT, CD11b, and CRP respectively [11].

$$\text{Sepsis} \quad \frac{e^{(-16.743+3.048\times\ln(PCT)+0.091\times\ln(CD11b)+0.381\times\ln(CRP))}}{1+e^{(-16.743+3.048\times\ln(PCT)+0.091\times\ln(CD11b)+0.381\times\ln(CRP))}}$$

Table 5. Logistic-regression analysis of biomarkers used for predicting neonates with and those without sepsis

	Coefficient	S.E.	Wald $\chi 2$	*P* Value	Odds Ratio (95% CI)
PCT	3.048	1.107	7.574	0.006	21.07 (95 CI:2.4-184.64)
CD11b	0.091	.032	7.908	0.005	1.1 (95 CI:1.03-1.17)
CRP	0.381	.192	3.935	0.047	1.46 (95 CI:1.01-2.13)
Constant	-16.743	5.189	10.410	0.001	0.000

P<0.05 is significant

Discussion

The diagnosis of neonatal sepsis is challenging as pediatricians are confronted with minimal non-specific clinical signs that often bear similarity to various noninfectious conditions [12]. Even blood cultures, the traditional gold standard for diagnosis, show high frequency of false negative results. Some mentioned causes are low density and intermittency in neonatal bacteremia, inadequacy of neonatal blood sampling, administration of empirical antibiotics before blood culture, which all contribute to the reduced sensitivity of the test [13]. Prompt diagnosis and treatment of neonatal sepsis can substantially reduce its high morbidity and mortality rates. In addition accurate diagnosis contributes to avoiding unnecessary antibiotic therapy which has been linked to the increased colonization of gram negative bacteria and the development of drug resistant strains [14].

When comparing the three markers, PCT, CRP and CD11b, PCT was associated with the highest AUC (0.923) followed by CD11b (0.912) and the least value was associated with CRP (0.883).

PCT had the highest AUC (0.923), with a corresponding sensitivity and specificity of 86.7 and 90.0 respectively and these results agree with previous studies reporting that the use of procalcitonin in the diagnosis of neonatal sepsis has proved to be very useful compared to other regular sepsis markers [15-17].

PCT had a sensitivity of 98.6% and a specificity of 88.9% in the diagnosis of neonatal sepsis [18] while other study reported that PCT showed both high sensitivity (100%) and specificity (100%) in the diagnosis of nosocomial sepsis [19]. These results agreed with another study which was carried out on neonatal cases with clinical sepsis and positive blood cultures reporting a sensitivity and specificity for PCT of 77% and 91%, respectively [20].

Meta-analysis done in 2011 indicated that PCT has a very good diagnostic accuracy for the diagnosis of neonatal sepsis. The area under the curve was 0.87, and the pooled sensitivity and specificity were 81and79%, respectively [17].

CD11b in another study, achieved a lower AUC (0.72) than the value measured in the current study (0.912) [21]. This can be easily explained by the lower values for sensitivity and specificity being 72% and 68% respectively in the same study.

In our study, CD11b showed that 85% sensitivity and 93.3% specificity were similar to those exhibited by other study that showed sensitivity and specificity of 86.3% and 100% respectively [22]. The same finding was reported by another case control study that found the sensitivity and specificity of CD11b to be 75% and 100% respectively [5].

It is important to determine appropriate cut-off values for diagnostic tests. The importance of determination of appropriate cut-off values was demonstrated by Yang et al. who found out that the sensitivity of neonatal sepsis diagnostic tests can be markedly increased by using optimized cut-off values compared with recommended cut-off values [23].

There are several criteria for determination of the most appropriate cut-off value in a diagnostic test with continuous results [24]. In this study, optimal cut-off values were calculated using shortest distance to the corner in ROC curve. The Youden index is another method used in many papers [25, 26]. Although in most tested biomarkers, both methods gave the

same cut-off points (data not shown), in other studies a difference appeared. For example, in CRP, using Youden index, the selected cut-off point was 10 which gave 75% and 100% sensitivity and specificity respectively, meanwhile the minimum distance to the corner method selected 8 which gave more balanced 77 and 93sensitivity and specificity respectively.

Although more than 170 biomarkers have been studied for use in evaluation of sepsis, no single test has been proved to be adequately specific and sensitive enough to be used as a gold standard or a stand-alone test for diagnosis of neonatal sepsis. Ideal sepsis biomarker would enable screening of patients at risk of sepsis, early diagnosis, monitoring response to intervention, and predicting outcome. To overcome the inadequacies of individual tests, physicians often rely on the results of multiple tests. However, the interpretation of multiple test results has the drawbacks of being subjective, empirical and need to be standardized [4].

A recent approach is to combine the results of multiple tests in an algorithm. This approach was originally introduced in the diagnosis of sepsis in adult patients [26-28], but more recently has been applied in neonatal sepsis [23, 30]. Most of these are algorithms that utilize logistic regression models in the interpretation of multiple test results. [28-30].

The importance of decision making based on combined test results grows more important and more feasible with the recent advances in artificial intelligence which give the computer the ability to make intelligent decisions that depend on previously entered data which can be derived from patient records stored in databases in modern hospitals. An early application of this approach was an android application that determines the probability of sepsis from clinical data such as temperature, respiratory rate, etc. using a logistic regression model [11].

In our study, we utilized a combination of CD11b, PCT, and CRP makers. Other studies have used different combinations as shown in Table (6). For example, Genel *et al* used combination of CD64, CD11b and CRP [30] while Yang et al. used combination of CD64, PCT, CRP and WBC [23].

In this study, logistic regression analysis was performed using CRP, procalcitonin and CD11b levels as predictor variables. A similar approach was performed for diagnosis of sepsis in adults. Combination of three biomarkers was used to calculate the predicted probability of sepsis by utilizing the derived equation from the multivariate logistic regression model [28].

Combining multiple test values were compared by using logistic regression method to the conventional pediatric systemic inflammatory response syndrome (SIRS) method. Logistic regression model achieved better sensitivity, specificity, and positive predictive values than individual tests alone [11].

In this study, the results showed that combined test (sepsis score) showed better ability to discriminate septic cases correctly (AUC=0.99; 95 CI=0.98-1; $P<0.001$), than either PCT alone (AUC=0.92; 95CI=0.87-0.98; $P<0.001$), CD11b alone (AUC=0.91; 95CI=0.85-0.98; $P<0.001$) or CRP alone (AUC=0.88; 95CI=0.81-0.95; $P<0.001$).
This superior discriminative power was obvious in other studies, For example a published study showed that the combination of CD64, CD11b and C reactive protein in the diagnosis of neonatal sepsis enhanced the sensitivity and negative predictive value (1.0 and 1.0 respectively) in CD64 alone (0.81 and 0.75), CD11b alone

(0.66 and 0.61) or CRP alone (0.81 and 0.77) [30]. In addition, a combination of CD64 and PCT improved sensitivity (90.9%) compared to nCD64 (79.5) alone or PCT alone (68.2%) [23].

This study demonstrated that the combination of the results of CRP, CD11b and, procalcitonin biomarkers could actually -in combination with clinical data -enhance the diagnostic discriminative power of traditional tests with higher accuracy thus leading to early diagnosis and treatment and better prognosis

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