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Research Article
Evaluation of New Biomarkers in Early Diagnosis of Neonatal Bacterial Infections

[1,2]Najah Fatou Coly Gueye, [5]Bruno Pereira, [2,3]Idrissa Bass, [3]Mbae Hamidou Ben Djabir, [3]Ndieme Ndiaye, [4]Julie Durif, [6]Souleymane Thiam, [6]Abdourahmane Samba, [6]Arame Ndiaye, [6]Idrissa Yaya Soumah, [6]Fatou Diedhiou, [6]Fatou Cissé, [7]Moustapha Djité, [7]Nene Oumou Kesso Barry, [7]Pape Matar Kandji, [7]Papa Madièye Gueye, [2,3]Ndeye Ramatoulaye Diagne Gueye, [8]Halimatou Diop Ndiaye, [6]Fatou Diallo Agne and [4]Vincent Sapin

[1]Laboratory of Medical biology, Diamniadio Children's Hospital, Senegal
[2]Medical School, University of Iba Der Thiam, Thies, Senegal
[3]Department of Pediatrics, Diamniadio Children's Hospital, Senegal
[4]Laboratory of Biochemistry and Molecular Biology, Gabriel Mont-Pied University Hospital, France
[5]Department of Clinical Research and Innovation, Clermont Ferrand University Hospital, France
[6]Laboratory of Medical Biochemistry, Faculty of Medicine, Pharmacy and Dentistry, Cheikh Anta Diop University, Dakar, Senegal
[7]Laboratory of Pharmaceutical Biochemistry, Faculty of Medicine, Pharmacy and Dentistry, Cheikh Anta Diop University, Dakar, Senegal
[8]Laboratory of Bacteriology and Virology, Faculty of Medicine, Pharmacy and Dentistry, Cheikh Anta Diop University, Dakar, Senegal

Abstract

Background and Objective: Bacterial neonatal infection is commonly encountered in Senegal. Management requires the use of specific and sensitive biomarkers. The identification of human alpha-defensins (HD-α) 1,2 and S100A12 and S100A8 proteins from amniotic fluid by proteomic analysis indicates the fetal response against an attack bacterial. The objective of this study was to evaluate these biomarkers for the early diagnosis of neonatal bacterial infections. **Materials and Methods:** Ninety-nine newborns were recruited from the pediatric ward of Diamniadio Children's Hospital in Senegal. The C-Reactive protein was measured using the latex immunoagglutination method. The S100 A8, A9, A12 and human neutrophil peptide 1, 2 and 3 proteins were determined by enzyme-linked immunoassay sandwich. Presepsin was assayed by chemiluminescent immunoassay with PATHFAST™. **Results:** Of the 99 newborns included, 20 had probable infection and 6 had definite infection. Death and complications were significantly higher in these groups. Among the new markers studied, presepsin showed substantial performance in diagnosis with a significant difference in concentration between the three defined groups ($p = 0.016$) with a sensitivity of 73.3% and a specificity of 76.2% at a threshold of 1079 pg mL^{-1}. The scenario was noticed for human neutrophil peptide-1 ($p = 0.011$) with an area under curve, sensitivity and specificity of 0.66, 66.7 and 73.9%, respectively at a maximum threshold of 2.77 ng mL^{-1}. The increase of S100 A8 protein can be considered as a risk factor for the occurrence of complications in subjects with confirmed neonatal bacterial infections. **Conclusion:** The study revealed that presepsin, HNP-1 and the S100A8 protein can help in the early diagnosis of neonatal bacterial infection, but also in management as a predictive marker of death and complications.

Key words: Biomarkers, S100 proteins (A8, A9, A12), human neutrophil peptide (HNP) 1,2,3, presepsin, neonatal bacterial infection, early diagnosis

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Corresponding Author: Najah Fatou Coly Gueye, Laboratory of Medical biology, Diamniadio Children's Hospital, Senegal

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Neonatal infection is the leading cause of neonatal mortality and hospitalization in developing countries[1].

Neonatal bacterial infection is classified into two groups: Early bacterial neonatal infection (EBNI) and late bacterial neonatal infection (LBNI)[2]. The BPNIs, which are transmitted ante or perinatally, occur most often in the first 72 hrs of life. The modes of infection are most often the transmembrane route (chorioamnionitis) with or without rupture of membranes and the ascending route (from the genital tract) during delivery and more rarely the transplacental hematogenous route[3]. Intrauterine infection is considered a pathological process that significantly increases the risk of early neonatal sepsis[4]. There are no pathognomonic signs to make the diagnosis. Biological markers are also used, but these do not allow a definite diagnosis. Decision-making algorithms have been developed using a combination of clinical signs and anamnestic criteria, particularly risk factors[1,5,6], to identify and manage any newborn at risk of infection. Blood culture is unanimously recognized as the reference test for the definitive diagnosis of BPN[5]. However, the ideal biomarker with high sensitivity and specificity is still a matter of controversy.

Research on biochemical markers for early diagnosis of neonatal infection has generated numerous publications. Indeed, the identification of human alpha defensins 1,2 and the proteins S100A12 and A100A8 in amniotic fluid by proteomic analysis signals the fetal response against bacterial aggression[7].

In Buhimschi's study[4], the protein profiles obtained revealed four biomarkers from which scores are calculated and these were human defensins 1 and 2, S100A12 and S100A8. Algorithms developed from the profiles obtained showed that parturient with a score of 4 were at risk of preterm delivery and early sepsis of the newborn[4,8,9]. It has been reported that human defensins 1 and 3 and the proteins S100A12 and S100A9 are part of the protein profile characteristic of inflammation and/or infection of the amniotic fluid[10].

The S100 A8, A9 and A12 proteins, also known as calgranulins A, B and C, are antimicrobials. The S100A8 and S100A9 proteins represent up to 40% of the cytosolic proteins of neutrophils, while S100A12 represents only 5%[11]. They are also expressed by monocytes, macrophages, platelets, epithelial and endothelial cells after cell stimulation[12,13]. The S100 proteins (A8, A9 and A12) in response to bacterial infection, LPS and monosodium urate (MSU) crystals, induce the recruitment, adhesion and release of neutrophils from the bone marrow. Therefore, they play an important role in the migration of the neutrophil to the inflammatory site[14,15]. In addition, S100A12 has chemotactic activity and is involved in the activation of intracellular signaling cascades leading to cytokine production and induction of oxidative stress[16,17].

Human alpha-defensins (HD-α) 1, 2 and 3 are also known as human neutrophil peptides HNP 1, 2, 3 (human neutrophil α-peptide) because they are exclusively secreted by neutrophils[18]. They belong to the family of antimicrobial peptides, which are part of the innate immune system activity[19]. They have a broad spectrum of antimicrobial activity (G- and G+ bacteria, fungi, enveloped viruses)[20]. The general mode of action of defensins is electrostatic binding of the cationic peptide to the external surface of the pathogen, followed by insertion of the peptide into the cytoplasmic membrane, resulting in leakage of the cellular contents into the extracellular medium[19]. The α-defensins 1 to 3 constitute 5-7% of the total protein content of human neutrophils and 30-50% of the total azurophilic granule content. They represent the most abundant antimicrobial peptides in neutrophils[21]. They activate bacterial phagocytosis by macrophages and stimulate the release of TNF-α and IFN-γ factors that act in an autocrine loop to amplify phagocytosis[22].

Another marker known to be a regulatory molecule of the adaptive immune system and a stimulator of phagocytosis by monocytes is being studied more and more. This is presepsin, which early increase during sepsis or other bacterial infections has made it a preferred test for laboratories[23]. Presepsin (sCD14-ST) is the subtype of sCD14, the soluble fragment of CD14[24]. It is released into the bloodstream by proteolysis and exocytosis. Presepsin levels increase within 2 hours and peak at 3 hrs after the onset of infection[25,26]. It rises earlier than interleukin-6 and procalcitonin, with a peak at 3 hrs and sustained elevation for at least 5 hrs[27]. The biological function of presepsin is not well described, however, it is considered a regulatory factor[27] and a stimulus for phagocytosis by monocytes[28].

Consequently, presepsin may modulate cellular and humoral immune responses by interacting directly with T and B cells and reduce the mortality rate caused by endotoxin shock and the severity of infections[29].

The aim of this study was to evaluate these biomarkers as early diagnostic markers in blood samples from neonates with suspected neonatal bacterial infection.

MATERIALS AND METHODS

Study type and setting: This is a descriptive and prospective study conducted between August, 2018 and May, 2020, firstly at the Pediatric Unit and the Laboratory of the Diamniadio Children's Hospital (HED), the Medical Biochemistry Laboratory of the Faculty of Medicine, Pharmacy and Odontology of the

Cheikh Anta Diop University in Dakar, Senegal and secondly at the Biochemistry and Molecular Genetics Laboratory of the Clermont Ferrand University Hospital in France.

Study population: The study was approved by the Research Ethics Committee of Cheikh Anta Diop University in Dakar. All participants gave written informed consent. A total of 99 newborns suspected of infection based on suggestive clinical symptomatology and anamnestic criteria were selected. According to the blood culture and CRP result at entry, the patients were divided into four groups:

- **Group 1 (G1):** Negative infection; when CRP <20 mg L^{-1} and blood culture negative
- **Group 2 (G2):** Probable infection defined by CRP ≥20 mg L^{-1} and negative blood culture
- **Group 3 (G3):** Definite infection for neonates with CRP ≥20 mg L^{-1} and positive blood culture
- **Group 4 (G4):** Combination of groups 2 and 3. Two subgroups are made within group G4, one for early bacterial infections before D7 (G4a) and another for late bacterial infections (G4b)

Subjects suspected of infection with the isolation of a contaminating germ (coagulase-negative *Staphylococcus*) and a CRP <20 mg L^{-1} were considered not infected. The same criteria were applied to those with a pathogen isolated on blood culture but with an inflammatory balance (CRP) <20 mg L^{-1}.

Sampling: Two samples were taken from the newborns on a dry tube and with sodium heparinate. These samples were then centrifuged at 3000 rpm. The CRP was immediately measured on the serum. The rest of the samples were stored with the plasma at -80°C and then transported in dry ice under the required conditions to the Biochemistry and Molecular Biology Laboratory of the Clermont Ferrand University Hospital.

Analytical methods: On plasma, presepsin was assayed with the PATHFAST™ automaton with reagents of lot 1202007350. The immunoassay is based on the principle of non-competitive chemiluminescence combined with MAGTRATION® technology.

The S100 proteins and α-defensin proteins are assayed with MyBioSource.com kits. The S100 A8, S100A9 and S100A12 proteins are assayed with the batch kits: L191219678, 0C095C and L6JQIECT9A, respectively. Proteins α-defensin 1, 2 and 3 are quantified with the respective batch kits: P18142336,02/2020 and P24142337. These kits use solid-phase sandwich ELISA principle.

Principle: The protein of interest is sandwiched between a specific antibody pre-coated on the plate and a specific antibody conjugated to biotin. This complex is then fixed by avidin conjugated to horseradish peroxidase (HRP). This enzyme, in the presence of a substrate TMB (3, 3', 5, 5' tetramethylbenzidine), a chromogen that produces a blue colour, oxidises it with hydrogen peroxide. The colour will then change to yellow when sulphuric or phosphoric acid is added to stop the reaction. Optical density is measured at wavelengths between 370 and 652 nm.

Absorbance is measured on a Tecan-Spark™ ELISA plate reader set at the wavelength specified by the supplier.

Data collection and statistical analysis: The data collected was analyzed using the Stata software (version 15, Stata Corp, College Station, United States). Qualitative variables are expressed as numbers and percentages. Quantitative variables are expressed according to the mean±standard distribution or as median and interquartile range. The Shapiro-Wilk test was used to validate normality (Gaussian distribution). Comparisons between groups (notably between the no infection/probable infection/certain infection groups and then between the infection yes/no groups) for parameters of a categorical nature were carried out by the Chi-square test or if necessary, by Fisher's exact test, followed, if necessary, by a *post hoc* test for multiple comparisons (Marascuilo procedure). The study of relationships between quantitative variables considered the estimation of Pearson's or Spearman's correlation coefficients, with respect to the statistical distribution of the variables under study. Comparisons between groups for quantitative variables were made by ANOVA or Kruskal-Wallis test if the conditions for applying ANOVA were not met. Homoscedasticity was investigated by Bartlett's test. If necessary (omnibus p-value less than 0.05), a *post hoc* test for pairwise comparisons was considered: Tukey-Kramer test post-ANOVA and Dunn test after Kruskal-Wallis test. These analyses were complemented in a second step by a ROC curve approach for which (i) Results are expressed in terms of AUC and 95% confidence intervals and (ii) Comparisons between AUCs were performed by the DeLong and DeLong's test. Intrinsic diagnostic values for sensitivity and specificity were then estimated for biomarker cut-offs determined with respect to biological relevance and indicators commonly reported in the literature (Youden, Liu and efficiency). Statistical tests were performed in a two-sided formulation for a 5% risk of error of the first kind.

RESULTS

In current study population composed of 99 neonates, six had proven sepsis (two had positive blood cultures for *Enterobacter* spp., one for *Escherichia coli*, one for *Staphylococcus saprophyticus* and two for *Staphylococcus aureus* and 20 had clinical sepsis.

Of these, six were older than 7 days and therefore had late neonatal bacterial infection (LNBI) and the rest had early infection (LNBI). There was a statistically significant difference in CRP concentration between the three groups (G1, G2 and G3; p = 0.001) but also between G1 and G4 (p = 0.001) (Table 1).

Among the maternal-fetal risk factors studied, there was no significant difference between the groups. The other risk factors studied in the mothers, such as age, number of gestation and parity, did not seem to be involved in the occurrence of neonatal infections. There was no significant difference between the study groups (Table 2).

The percentage of deaths was significantly higher for group 3 and group 4 with p = 0.001 and p<0.001, respectively. The same trend was observed with complications (p = 0.001 and p<0.001). The general condition seems to be worse in subjects with confirmed NBI with a statistically lower percentage of good general condition in group 4 (15%, p = 0.016) (Table 3).

Presepsin was significantly higher in group 4 than in group 1 (p = 0.0042) (Table 4). Group 2 had higher presepsin concentrations (1975.5 mg L^{-1}) than the others. In contrast, CRP was significantly higher in group 3. Regarding S100 proteins, S100 A8 was slightly higher in the subjects with definite infection and although trends were observed, their difference was not significant (p = 0.07). As for S100 A9, its concentration was significantly lower in neonates with confirmed sepsis than in those without (p = 0.022). The concentration of HNP-1 was significantly higher in the infected group.

The CRP is significantly higher in subjects with ENBI (p = 0.0001) and LNBI (p = 0.0001) than in uninfected subjects. However, the concentration is higher in neonates with LNBI than in those with ENBI with a mean of 42.4 (25.1, 54) mg L^{-1} and 115.5 (62.7, 190) mg L^{-1}, respectively. Statistically significant differences were also found with presepsin regardless of the type of infection with p = 0.034 for ENBIs and p = 0.011 for LNBIs (Table 5).

In the total population, presepsin is higher in subjects with complications (p = 0.001). The S100 A8 is significantly higher in neonates with complications and those who died with p = 0.0002 and p = 0.005, respectively. The same is true for CRP with p = 0.002 and p = 0.0001, respectively (Table 6).

Table 1: Group allocation criteria for the 99 newborns

	G1 (Not infection) (CRP<20 and Germs -)	G2 (Probable infection) (CRP>20 and Germs -)	G3 (Infected) (CRP>20 and Germs +)	p-value	G4 (CRP>20 and Germs +or-)	p G1 vs G4
Number of patients (n)	73	20	6		26	
CRP (mg L^{-1})	2 (0, 3)	46 (23, 69)	52 (25, 192)	0.001	48 (25, 75)	0.001
Isolated pathogens n (%)	2 (2%)	2 (10%)	5 (83%)	0.001	7 (78%)	0.001

Table 2: Maternal characteristics and risk factors

	G1 No infection	G2 Probable infection	G3 Infected	p-value	G4	p G1 vs G4
Domiciliation (in dakar and outside dakar)	20/73 (27 %)	8/20 (40%)	2/6 (33%)	ns (0.503)	10/26 (38%)	ns (0.292)
Maternal age (n/total and %)				ns (0.571)		ns (0.431)
<18 years	6/71 (8.45%)	2/18 (11.1%)	6/71 (8%)		2/24 (8%)	
between 18 et 35 years	55/71 (77%)	12/18 (66.7%)	4/6 (66.7%)		16/24 (66 %)	
>35 years	10/71 (11%)	4/18 (22%)	2/6 (33.3%)		6/24 (25%)	
Number of pregnancies						
Number of successful pregnancies (G)	2 (1, 5)	3 (1, 4)	3 (4, 2)	ns (0.752)	3 (1.5, 4)	ns (0.459)
Regular pregnancy monitoring (P)	2 (1, 4)	3 (1, 4)	3 (3, 2)	ns (0.704)	3 (1.5, 3.5)	ns (0.403)
Tracking parameters						
Number of prenatals consultation	4 (3, 4)	4 (3, 4)	4 (4, 4)	ns (0.71)	4 (3, 4)	ns (0.766)
Number of ultrasounds	1 (1, 2)	1 (1, 2)	1 (1, 2)	ns (0.945)	1 (1, 2)	ns (0.743)
Toxoplasmosis (n/total and %)	60/72 (83%)	18/20 (90%)	4/6 (67.7%)	ns (0.331)	4/26 (15%)	ns (0.880)
Rubella (n/total and %)	61/72 (84.7%)	19/20 (95%)	5/6 (83%)	ns (0.472)	2/26 (7%)	ns (0.504)
HIV (n/total and %)	24/73 (32.8%)	10/20 (50%)	1/6 (16.7%)	ns (0.224)	15/26 (57%)	ns (0.475)
Syphilis (n/total and %)	30/73 (41.1%)	9/20 (45%)	3/6 (50%)	ns (0.823)	14/26 (53%)	ns (0.653)
Vaccination Incomplete (n/total and %)	48/72 (66.7%)	12/19 (63.2%)	4/6 (66.7%)	ns (0.929)	16/25 (64%)	ns (0.811)

ns: Non-significative

Table 3: Parameters and follow-up of hospitalization of newborns according to their infectious status

	G1 No infection	G2 Infection Probable	G3 Infected	p-value	G4	p G1 vs G4
Full term delivery (n/total and %)	64/73 (87.6%)	18/20 (90%)	5/6 (83.3%)	ns (0.872)	23/26 (88.4%)	1
Place of birth	20/73 (27.4%)	8/20 (40%)	2/6 (33.3%)	0.503	10/26 (38.4%)	0.292
Anthropometric parameters						
Sex ratio (H/F)	36/37	14/06	02/04	ns (0.144)	16/10	0.284
Early infection (<7 days) (n/total and %)	58%	25%	17%	ns (0.241)	23%	0.079
Birth weight (g)	2680 (2200, 3000)	3200 (2800, 3600)	2865 (2700, 3200)	0.03	3000 (2700, 3500)	0.009
Birth size (cm)	48 (46, 50)	49 (45, 51)	48.75 (45, 51)	ns (0.928)	49 (45, 51)	0.715
Reasons for admission to pediatrics						
Respiratory distress/asphyxia/dyspnea	39/73 (53%)	15/20 (75%)	3/6 (50%)	ns (0.206)	69%	ns (0.161)
Cyanosis	14/73 (19.2%)	5/20 (25%)	1/6 (16.2%)	ns (0.817)	6/26 (23%)	ns (0.777)
Fever	29/72 (40%)	7/20 (35%)	4/6 (66.7%)	ns (0.639)	11/26 (42.3%)	ns (0.559)
Refusal to breastfeed	25/73 (34%)	7/20 (35%)	2/6 (33.3%)	ns (0.997)	9/26 (34.6%)	ns (0.973)
Prematurity	3/73 (4%)	3/20 (15%)	0	ns (0.198)	3/26 (11%)	ns (0.173)
Jaundice	4/73 (5.48%)	0	0	ns (0.67)	0	ns (0.223)
Good general condition (n/total and %)	31/73 (42.5%)	3/20 (15%)	1/6 (16.7%)	0.046	4/26 (15%)	0.016
Apgar score at 5 min	7 (5, 8)	6 (5, 8)	7.5 (6, 8)	ns (0.952)	6 (5, 8)	ns (0.99)
Apgar score at 10 min	9 (6.5, 9)	8 (7, 10)	8 (8, 9)	ns (0.886)	9 (7, 9)	ns (0.645)
Outcome of hospitalization						
Deaths (n/total and %)	7/64 (11%)	8/20 (40%)	3/5 (60%)	0.001	11/25 (44%)	p<0.001
Transfer (n/total and %)	2/57 (3%)	1/12 (8.3%)	0	ns (0.488)	1/14 (7%)	ns (0.488)
Complications (n/total and %)	13/65 (20%)	12/19 (63%)	3/6 (50%)	0.001	15/25 (60%)	p<0.001
Number of days hospitalization	10 (7, 16)	10.5 (6, 26.5)	4 (3, 23)	ns (0.821)	10 (4, 24)	ns (0.9)

ns: Non significative

Table 4: Comparison of biological markers between different groups

	G1 No infection	G2 Probable infection	G3 Infected	P (3 groups)	G4 Infected and probable infection	p G1 vs G4
Number of patients (n)	73	20	6		26	
CRP (mg L^{-1})	2 (0, 3)	46 (23, 69)	52 (25, 192)	0.0001	48 (25, 75)	0.0001
Presepsin (pg mL^{-1})	717.5 (480, 1075)	1975.5 (603, 2310)	1079 (1422, 1484)	0.016	1745 (603, 2310)	0.0042
S100A8 (ng mL^{-1})	59.7 (30.4, 82)	70.8 (41.3, 111.59)	74.65 (49.2, 89.4)	0.194	73.4 (43.2, 100)	0.072
S100A9 (ng mL^{-1})	5.025 (1.19, 9.62)	3.03 (0.039, 6.41)	0.859 (0.039, 2.39)	0.022	2.01 (0.039, 5)	0.02
S100A12 (ng mL^{-1})	1000 (821.3, 1000)	1000 (611.31, 1000)	975.07 (496.61, 1000)	0.608	1000 (581.91, 1000)	0.336
HNP-1 (ng mL^{-1})	0.8 (0.31, 3.38)	2.905 (0.31, 6.47)	22.77 (15.04, 31.73)	0.011	4.14 (0.31, 22.77)	0.028
HNP-2 (pg mL^{-1})	129.46 (69.55, 186.08)	116.68 (84.1, 194.49)	126.4 (39.53, 156.82)	0.918	118.82 (83.02, 193.6)	0.938
HNP-3 (ng mL^{-1})	2.065 (0.23, 5.57)	2.92 (0.23, 12.4)	75.95 (75.95, 75.95)	0.235	3 (0.23, 13.09)	0.269

Table 5: Comparison of biological markers according to the type of infection

	G4a Early infection (<7 days)	p-value (G1 vs G4a)	G4b Late infection (>7 days)	p-value (G1 vs G4a)
Number of patients (n)	19		7	
CRP (mg L^{-1})	42.4 (25.1, 54)	0.0001	115.5 (62.7, 190)	0.0001
Presepsin (pg mL^{-1})	1453 (573, 2192)	0.034	1987 (1964, 4998)	0.011
S100A8 (ng mL^{-1})	63 (41.3, 94.7)	0.223	86.8 (64.1, 150)	0.074
S100A9 (ng mL^{-1})	1.37 (0.04, 5.70)	0.043	3.03 (2.48, 3.09)	0.158
S100A12 (ng mL^{-1})	950.14 (496.61, 1000)	0.19	1000 (1000, 1000)	0.671
HNP-1 (ng mL^{-1})	4.14 (0.31, 31.73)	0.077	4.54 (2.47, 14.45)	0.086
HNP-2 (pg mL^{-1})	112.54 (72.48, 163.5)	0.544	193.6 (135.32, 195.39)	0.126
HNP-3 (ng mL^{-1})	3 (0.235, 44.25)	0.292	7.02 (0.94, 12.74)	0.607

Table 6: Factors influencing deaths and complications in general population

	Death			Complication		
	No	Yes	p-value	No	Yes	p-value
Number of patients (n = 88)	71	17	61	28		
CRP (mg L^{-1})	2 (0, 6)	20.8 (2.9, 52.8)	0.002	2 (0, 6)	14 (3, 52)	0.0001
Presepsin (pg mL^{-1})	751 (505, 1484)	1694.5 (640, 3246)	0.063	697 (484, 1035)	1484 (605, 2074)	0.001
S100A8 (ng mL^{-1})	49.8 (31.1, 84.7)	87.05 (59.7, 135.5)	0.005	48.6 (30.4, 72.8)	89.4 (49.8, 150)	0.0002
S100A9 (ng mL^{-1})	4.02 (1.13, 8.04)	2.48 (0.039, 15.26)	0.692	3.75 (0.58, 8.67)	3.52 (0.53, 6.41)	0.626
S100A12 (ng mL^{-1})	1000 (569.08, 1000)	1000 (680.03, 1000)	0.43	1000 (585.65, 1000)	1000 (934.59, 1000)	0.393
HNP-1 (ng mL^{-1})	2.165 (0.31, 6.47)	2.43 (0.31, 9.36)	0.992	0.8 (0.31, 6.14)	2,90 (1.14, 7.74)	0.125
HNP-2 (pg mL^{-1})	127.12 (68.22, 193.6)	118.1 (85.22, 170.18)	0.969	118.82 (69.88, 185.47)	119.76 (65.98, 194.49)	0.953
HNP-3 (ng mL^{-1})	2.27 (0.235, 10.47)	1.62 (0.23, 4.13)	0.607	2.28 (0.23, 5.73)	3.64 (0.23, 10.93)	0.515

In the population with definite infection, only S100A8 is significantly higher in subjects with death and complications (p = 0.004 and p = 0.0009) (Table 7).

The CRP has a high sensitivity and specificity of 88 and 99% with an NPV and PPV of 96%. In addition, S100A9 and HNP-1 also have good sensitivity (68, 66.7%) and specificity (67.1, 73.9%). Their PPVs are relatively high (42.5, 53.8%). The S100A8 has a good sensitivity of 80.8% with a low specificity of 33.3% and an NPV of 82.1% (Table 8).

The S100 A8 is correlated with presepsin in the general population but also in group 4 with a stronger correlation in the latter group, r = 0.729 and p = 0.0020. The S100 A12 is also correlated with S100 A8 in the general population and group 4 with respectively, r = 0.251 (p = 0.0213) and r = 0.408 (p = 0.048). The HNP-3 and HNP-1 are strongly correlated in the general population and in group 4, r = 0.778 (p<0.001) and r = 0.661 (p = 0.01). However, HNP-1 is inversely correlated with S100 A12 with r = -0.64 (p = 0.0016). In the general population, CRP is strongly and significantly correlated with presepsin r = 0.589 (<0.001) and moderately correlated with S100A8 and HNP-1 with r = 0.282 (0.0057) and r = 0.419 (0.0004), respectively (Table 9).

The AUC of presepsin is significantly higher compared to S100 A8 and S100A12 with p = 0.036 and p = 0.0023, respectively. However, the AUC of CRP is significantly higher than all other parameters (Table 10).

All newborns with suspected INB should have CRP and presepsin measured. In the case of BPN, a decrease in S100 A9 protein would help in the diagnosis. The risk of complications should be monitored by measuring the S100 A8 protein (Fig. 1).

In terms of sensitivity and specificity, CRP can be considered the best marker. However, S100 A8 protein has good sensitivity and presepsin good specificity (Fig. 2).

Table 7: Factors influencing deaths and complications in G4 population

	Death			Complication		
	No	Yes	p-value	No	Yes	p-value
Number of patients (n = 26)	14	11		10		
CRP (mg L^{-1})	50 (24, 155)	48 (25, 62)	0.742	48 (25, 155)	52 (24, 75)	0. 697
Presepsin (pg mL^{-1})	1614.5 (543, 1987)	2778 (1694.5, 4122)	0.066	1012.5 (484, 1745)	1987 (1484, 2736)	0.077
S100A8 (ng mL^{-1})	46.85 (38.7, 80)	100 (77.5, 150)	0.004	41.3 (36, 64.1)	89.4 (77.5, 150)	0.0009
S100A9 (ng mL^{-1})	2.71 (0.85, 5.04)	1.34 (0.039, 5)	0.558	0.44 (0.039, 1.68)	2.78 (1.01, 5.04)	0.057
S100A12 (ng mL^{-1})	967.29 (552.51, 1000)	1000 (657.36, 1000)	0.614	705.57 (496.61, 1000)	1000 (1000, 1000)	0.072
HNP-1 (ng mL^{-1})	4.83 (2.435, 23.4)	2 (0.31, 18.905)	0.418	6.47 (2.91, 23.38)	2.905 (0.31, 13.45)	0.315
HNP-2 (pg mL^{-1})	128.78 (112.41, 211.9)	110.5 (51.23, 156.82)	0.16	116.68 (85.18, 131.17)	123.86 (82.83, 193.6)	0.907
HNP-3 (ng mL^{-1})	6.65 (0.23, 13.09)	1.62 (0.23, 5.115)	0.322	10.47 (2.84, 44.25)	0.94 (0.23, 9.81)	0.203

Table 8: Sensitivity and specificity of inflammation proteins in the diagnosis of neonatal bacterial infections

Markers	AUC (95% CI)	Max threshold	Sensitivity (95% CI)	Specificity (95% CI)	PPV (%)	NPV (%)
CRP (mg L^{-1})	0.94 (0.90, 1)	16.3	88% (70, 98%)	99% (93, 100%)	96	96
Presepsin (pg mL^{-1})	0.75 (0.58, 0.91)	1079	73.3% (44, 92%)	76.2% (60, 87%)	52.4	88.9
S100A8 (ng mL^{-1})	0.58 (0.49, 0.74)	31.25	80.8% (60.6, 93.4%)	33.3% (22.4, 45.7%)	31.3	82.1
S100A9 (ng mL^{-1})	0.65 (0.53, 0.77)	3.16	68% (46.5, 85.1%)	67.1% (54.9, 77.9%)	42.5	85.5
S100A12 (ng mL^{-1})	0.43 (0.31, 0.55)	496.6	83.3% (62.6, 95.3%)	18% (9.36, 30%)	28.6	73.3
HNP-1 (ng mL^{-1})	0.66 (0.52, 0.81)	2.77	66.7% (43, 85.4%)	73.9% (58.9, 85.7%)	53.8	82.9
HNP-2 (ng mL^{-1})	0.50 (0.37, 0.63)	126.4	48% (27.8, 68.7%)	48.5% (36.2, 61%)	25.5	71.7
HNP-3 (ng mL^{-1})	0.59 (0.42, 0.77)	2.795	60% (32.3, 83.7%)	68.4% (51.3, 82.5%)	42.9	81.3

PPV: Positive predictive value and NPV: Negative predictive value

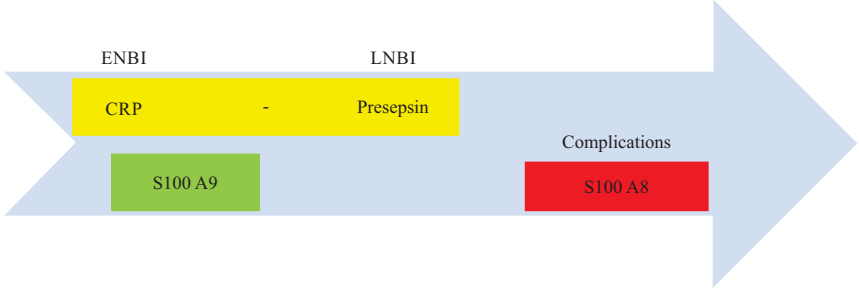

Fig. 1: Decision algorithm by type

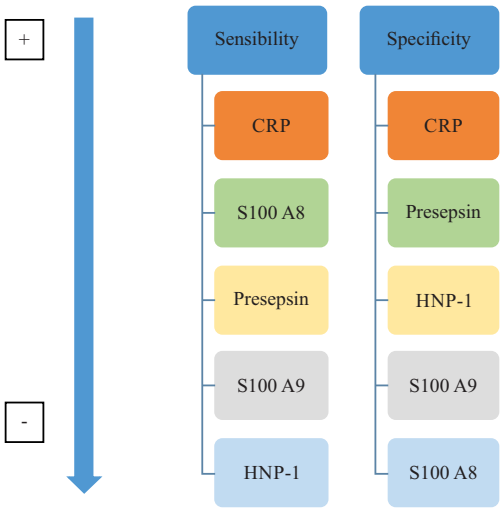

Fig. 2: Algorithm for the choice of markers in the diagnosis of neonatal bacterial infections according to their sensitivity and specificity

Table 9: Correlations of markers in general population and G4 group

	CRP	Presepsin	S100A8	S100A9	S100A12	HNP-1	HNP-2
General population r (p-value)							
Presepsin	**0.589 (<0.001)**	1.000					
S100A8	**0.282 (0.0057)**	**0.349 (0.0078)**	1.000				
S100A9	-0.194 (0.059)	0.033 (0.80)	-0.121 (0.24)	1.000			
S100A12	0.008 (0.94)	0.200 (0.15)	**0.251 (0.0213)**	**0.234 (0.0308)**	1.000		
HNP-1	**0.419 (0.0004)**	**0.368 (0.01)**	0.213 (0.08)	-0.098 (0.43)	-0.193 (0.013)	1.000	
HNP-2	0.104 (0.31)	0.158 (0.025)	**0.500 (<0.001)**	0.020 (0.66)	0.205 (0.28)	0.136 (0.28)	1.000
HNP-3	0.228 (0.10)	0.206 (0.25)	0.016 (0.91)	-0.015 (0.91)	0.103 (0.47)	**0.778 (<0.001**)	0.017 (0.90)
G4 Population r (p-value)							
Presepsin	0.281 (0.31)	1.000					
S100A8	0.072 (0.72)	**0.729 (0.0020)**	1.000				
S100A9	-0.063 (0.27)	0.335 (0.22)	0.173 (0.40)	1.000			
S100A12	0.234 (0.94)	0.250 (0.36)	**0.408 (0.048)**	0.255 (0.22)	1.000		
HNP-1	0.190 (0,40)	0.000 (1)	-0.073 (0.75)	-0.116 (0.61)	**-0.644 (0.0016)**	1.000	
HNP-2	0.011 (0.95)	-0.235 (0.41)	0.127 (0.54)	0.120 (0.57)	0.143 (0.51)	0.032 (0.89)	1.000
HNP-3	-0.196 (0.48)	0.293 (0.44)	-0.051 (0.85)	-0.115 (0.68)	-0.262 (0.34)	**0.661 (0.01)**	0.204 (0.46)

CRP: C-Reactive protein, S100A8A,9 or A12: S100 proteins A8, A9 or A12, HNP- (1,2 or 3): Human neutrophil peptide (1,2 or 3), Strong correlations ($r \geq 0.5$) and Weak correlations ($0.25 \leq r < 0.5$) and significant correlations are highlighted in bold

Table 10: Comparison of the AUC of presepsin and CRP with other markers

Markers	Number of patients	AUC	p-value
Presepsin/S100A8	57	0.75/0.58	0.036
Presepsin/S100A12	52	0.75/0.43	0.0023
Presepsin/HNP-1	44	0.74/0.70	0.74
Presepsin/HNP-2	53	0.72/0.53	0.13
Presepsin/HNP-3	32	0.73/0.65	0.59
Presepsin/CRP	57	0.75/0.94	0.008
CRP/S100A8	95	0.96/0.62	<0.001
CRP/S100A12	85	0.96/0.43	<0.001
CRP/HNP-1	67	0.96/0.67	<0.001
CRP/HNP-2	93	0.96/0.51	<0.001
CRP/HNP-3	53	0.95/0.60	<0.001

AUC: Area under curve

DISCUSSION

This study confirmed that clinical signs alone are not sufficient to make a diagnosis of NBI. Indeed, the clinical signs classically collected are not different in our study, except for the general condition which is worse in infected subjects (Table 3). However, the percentage of deaths is significantly higher in group 3 and group 4. While the percentage of complications shows a significant difference between the three groups and between G1 and G4. The maternal-fetal risk factors studied also had no influence on the occurrence of bacterial infections in the newborn (Table 2).

In case of bacterial infection, the immune system of the newborn is the first line of resistance. Therefore, antimicrobial peptides can fight infections through their direct microbicidal properties and/or indirectly through their influence on host immune responses[30]. The HNP1 by binding to lipid II, a synthetic peptidoglycan exerts antimicrobial activity[31]. When it comes to S100 proteins, their release is caused by cellular stress and the activation of phagocytes such as neutrophils and macrophages. By binding to pattern recognition receptors such as TLRs and RAGE, the released S100 proteins activate immune cells and endothelial cells and become danger signals[32,33]. Presepsin, that is a truncated shape of soluble CD14 (13 kDa), is released by proteolytic cleavage on activated monocytes[34]. Its physiological variations in the newborn are not clearly defined[35]. Of the 99 neonates selected, six had a bacterial infection confirmed by a positive blood culture (Table 1). *Enterobacter* spp. and *Staphylococcus aureus* were isolated separately from two newborns, *Escherichia coli* and *Staphylococcus saprophyticus* from one subject each.

Of the studied markers, presepsin and CRP show significantly different concentrations between the three groups but also between group 1 and group 4 (Table 4). The CRP was higher in the definite infection group with a median of 52 mg L^{-1} and ranged from 25 to 192 mg L^{-1} while presepsin was higher in the probable infection group with a median of 1975.5 pg mL^{-1} and ranged from 603 to 2310 mg L^{-1} (Table 4).

Consequently, CRP and presepsin are early diagnostic tools regardless of the type of infection (EBNI and LBNI) (Table 5). We found statistically significant differences in median CRP and presepsin values ($p = 0.0001$ and 0.001) in subjects with complications (Table 6).

In several studies, presepsin has emerged as a potential and accurate biomarker for the diagnosis of early and late neonatal sepsis[36-38]. Thus, a significant increase in presepsin may indicate a strong activation of the innate immune response in the newborn with a risk of sepsis. In the meta-analysis by van Maldeghem[39], it is shown that very high concentrations of sCD14-ST were found in septic neonates compared to healthy controls with higher levels in LBNI compared to EBNI. This would explain our results in the definite infection group with lower levels than in the probable infection group as this group is 75% LBNI. Although the mechanism of the physiological variation in plasma presepsin levels in the neonatal period is unknown, the concentrations found in healthy neonates at birth were not as high as those in neonates with sepsis[35]. Presepsin ranged from 99.2 pg mL^{-1} to 1180 pg mL^{-1} at birth and 129.0 pg mL^{-1} to 655 pg mL^{-1} on day one[35]. However, according to Pugni *et al.*[38], the reference ranges for presepsin in neonates were much higher than those observed in healthy adults. The study of Davide in adults showed that there was no significant difference by sex[40]. However, presepsin ranges with inside the first 28 days of lifestyles appear probably to be tormented by the form of delivery, gestational and postnatal age, delivery weight and the presence of respiration misery syndrome or maternal chorioamnionitis[41].

The S100 A9 is statistically lower in newborns with definite infection 0.859 ng mL^{-1} with a minimum of 0.039 ng mL^{-1} and a maximum of 2.39 ng mL^{-1} ($p = 0.022$) (Table 4). It appears to be of interest in the diagnosis of EBNI (Table 5) with a p-value of 0.043. Unlike the S100 A8 protein which is higher in neonates with definite infection but without any significant difference between the three groups ($p = 0.194$) and between group 1 and group 4 ($p = 0.072$) (Table 4).

In healthy newborns, high serum concentrations of the endogenous toll-like receptor 4 ligands of S100A8 and S100A9 induce a state of microbial hyporeactivity in blood monocytes, also known as stress tolerance[42]. The S100A8 and S100A9 are expressed separately but the proportion of S100A8 is higher than that of S100A9. In the absence of its binding partner S100A9, serum levels of S100A8 are almost undetectable[43]. They often exist as homodimers, S100A8/A9 heterodimers called calprotectin or heterotetramers (S100A8/A9)4[44]. It is recognized that the more stable heterodimer form is responsible for most biological interactions and that intestinal deficiency of the latter in newborns increases the risk of developing intestinal dysbiosis and other associated adverse conditions[42]. However, our study showed that individually, S100 A8 and S100A9 proteins may have an activity.

The elevation of S100 A8 in the infected group is related to the observed complications and deaths with $p = 0.0009$ and 0.004, respectively (Table 7). This observation was confirmed in the general population, where S100 A8 protein is significantly higher in subjects who died or had complications with $p = 0.005$ and $p = 0.0002$, respectively (Table 6). As a result, in assessing the occurrence of complications and death in newborns with suspected neonatal infection, S100 A8 would play an important role.

For S100 A12, the threshold of the calibration range was too low (1000 ng mL^{-1}). Thus, a mean of 1000 ng mL^{-1} is obtained for each group without significant difference (Table 4). Nevertheless, its variations were already studied in newborns and according to Tosson's study[45], this variation, as well as those of IL-6 and IL-10, were significant ($p<0.001$) between the infected and control groups[45]. For the uninfected group, S100A12 had a median of 180 ng mL^{-1} and ranged from 26-1110 ng mL^{-1} while in the probable infection group, the median was 400.00 ng mL^{-1} (34.0-2380.0) ng mL^{-1}. Finally, the confirmed infection group had a median of 360 ng mL^{-1} (79-1700) ng mL^{-1}. The concentrations in the probable infection group were higher.

For α-defensins, HD α1 concentrations were significantly higher in group 3 than the others with a p-value equal to 0.011 (Table 4). On the other hand, the variations between the three groups of HDs α2, are not significant with a non-significant difference ($p = 0.918$) but also between G1 and G4 ($p = 0.938$). For HD α3, a very significant variation was observed between the three groups ranging from 2.065 ng mL^{-1} for G1 to 75.95 ng mL^{-1} for G3 but without significance ($p = 0.235$) (Table 4).

These results were inconsistent with those in the study of Hoover *et al.*[46], which found higher concentrations of HNP 1-3 in subjects with bacterial infections compared to uninfected subjects with a good correlation with neutrophils ($r = 0.988$, $p = 0.0001$)[47]. Even preterm newborns with pneumonia had higher concentrations of HNP1-3 than controls, demonstrating that they can activate an innate immune system[21]. Indeed, defensins have microbicidal activities, when leukocytes ingest pathogens into phagocytic vacuoles, the granules fuse to these vacuoles and deliver their contents to the target microbe[18]. Defensins act on microbial membranes as a multimer that can form membrane pores[21]. In our study, HD α1 appears to have a more pronounced antibacterial activity than the other defensins studied. Indeed, in relation to the type of infection, trends seem to emerge with $p = 0.077$ for INBPs and $p = 0.086$ for LBNIs (Table 5). However, in most studies[48-50] it is the total concentration of HNPs 1-3 that is investigated and it is important to take them individually to understand their involvement in infections. In our study, we determined the α-defensins separately.

The S100 A8 protein is positively correlated with presepsin $r = 0.349$ ($p = 0.0078$) (Table 9) in the general population. This correlation is even stronger in group 4, $r = 0.729$ ($p = 0.0020$). The S100 A12 protein is also positively correlated in both groups with respectively, $r = 0.251$ ($p = 0.0213$) and $r = 0.408$ ($p = 0.048$) to S100 A8 as well as to S100 A9 in the general population $r = 0.234$ ($p = 0.0308$) (Table 9). The HNP-1 is strongly correlated with HNP-3 with $r = 0.778$ ($p<0.001$) and $r = 0.661$ ($p = 0.01$) in the general population and group 4, respectively. On the other hand, it is negatively correlated with S100 A12, $r = -0.644$ ($p = 0.0016$). In the general population, HNP-1 is correlated with CRP ($r = 0.419$ ($p = 0.0004$) and presepsin ($r = 0.368$ ($p = 0.01$)) (Table 9). The observed correlations confirm the relevance of presepsin, CRP, S100A8 and HNP-1 in the study. The CRP had the best sensitivity and specificity (Se = 88% (70, 98%) and Spe = 99% (93, 100%)) compared to the other parameters with an NPV and PPV equal to 96% (Table 8). It is followed by presepsin with a sensitivity of 73.3% ranging from 44 to 92% and a specificity of 76.2% (60, 87%). Its PPV and NPV are 52.4 and 88.9%.

In a meta-analysis of eleven studies[51], with a total number of 783 newborns, the pooled sensitivity of serum presepsin for prediction of neonatal sepsis was 0.91 (95% CI (0.87-0.93)) and the pooled specificity was 0.91 (95% CI (0.88-0.94)). The diagnostic odds ratio was 170.28 (95% CI (51.13-567.11)) and the area under the curve (AUC) was 0.9751[51].

Comparison of AUCs showed that CRP and presepsin are good diagnostic markers (Table 10).

Ultimately, in view of these results, two decision algorithms (Fig. 1 and 2) can be drawn up according to the type of infection and the sensitivity and specificity of the markers for the early diagnosis of bacterial neonatal infections.

The combined assay of CRP and presepsin allows early diagnosis of both early and late neonatal bacterial infection. In follow-up, measurement of the S100 A8 protein can predict the onset of complications (Fig. 1). In terms of specificity and sensitivity, CRP is clearly the best choice (Fig. 2).

The limitations remain the small volume of sample collection to avoid blood spoliation of newborns and the range of the kit (low concentration) of S100 A12 protein did not allow significant results.

CONCLUSION

The evaluation of these biomarkers showed that presepsin is a good marker for early diagnosis of neonatal infections. The S100 A8 is a risk factor for complications and death. However, further studies are needed to confirm the individual activity of S100 A9 proteins in bacterial infections and their use as a diagnostic marker for neonatal bacterial infections as well as for S100 A12. For defensins, HD α1 seems to be more promising than HD α2 and HD α3.

SIGNIFICANCE STATEMENT

The incidence of neonatal mortality is very high and among the causes, neonatal infections, particularly bacterial. By 2030, all countries aim to reduce neonatal mortality to below 12 deaths per 1,000 live births and under-five mortality to below 25 deaths per 1,000 live births. The aim of this study was to identify new biomarkers of early diagnosis for neonatal bacterial infections. Some biomarkers were evaluated which are secreted by innate immunity during the infection. The results showed us that even if CRP is a good marker, HNP-1 and S100A8 are interesting and require further study.

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