Diplomarbeit

IL-6 as diagnostic marker for neonatal sepsis – alone and in combination with other inflammatory markers

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Graz, am 24.02.2023 *Julia Eichberger eh.*

Preface

This diploma thesis offers an overview of my own original scientific work. The included papers have been published in “Frontiers in Pediatrics” before the finalization of this thesis. To avoid copyright infringement and self-plagiarism I asked “Frontiers in Pediatrics” for the right to incorporate my publications into this thesis. The journal’s publisher granted me the right to include the following papers in my diploma thesis, provided that proper credit is given to the original source. The corresponding author has been informed about my intention and agrees to the inclusion of the papers.

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I dedicate this thesis to my beloved daughter Emilia.

Abstract

Background: Neonatal sepsis is a major cause of morbidity and mortality in both preterm and term infants. Early onset neonatal sepsis (EONS) presents within the first 72h of life, while late onset sepsis (LOS) occurs after this time. Diagnosis is difficult as signs and symptoms are nonspecific, and routinely used laboratory tests can neither confirm nor rule out neonatal sepsis. Inflammatory markers are widely used as an additional diagnostic tool. Interleukin-6 (IL-6) is part of the fetal inflammatory response syndrome (FIRS) and therefore an interesting early marker for neonatal sepsis.

Methods: IL-6 diagnostic accuracy studies for diagnosing neonatal sepsis published between 1990 and 2020 were retrieved using the PubMed database and systematically reviewed. IL-6 was analysed as a sole marker and in combination with other inflammatory markers for the diagnosis of early onset sepsis. The diagnostic potential of IL-6 was correlated with gestational age, timing and site of sample collection. Sensitivity and specificity values and positive and negative predictive values of IL-6 were reported and subgroup analyses performed. The quality of IL-6 diagnostic accuracy studies was assessed using a STARD checklist adapted for neonates with neonatal sepsis.

Results: We identified 31 studies on IL-6 diagnostic accuracy for EONS diagnosis between 1990 and 2020 including a total of 3276 infants. The range of IL-6 sensitivity and specificity in neonatal samples was 42.1% to 100% and 43% to 100%, the median values were 83% and 83.3%, respectively. IL-6 accuracy was better in preterm infants than in mixed study populations. The sensitivity and specificity in umbilical cord blood was higher than in neonatal peripheral blood, 83% vs 71% and 85% vs 77% respectively. The combination of IL-6 and CRP had a sensitivity in the range of cord blood IL-6 as single measure (84% vs 83%), but far lower specificity (61% vs 85%).

Conclusion: IL-6 is a good diagnostic marker of EONS within a study population of preterm infants, with best results for cord blood IL-6 using cut-off values above 30 pg/mL. The biomarker combination of IL-6 and CRP was found to be highly sensitive, but poorly specific. Optimisation of cut-off values, timing of sample collection and positivity criterion of the test could improve the diagnostic value of biomarker combinations.

Kurzfassung

Hintergrund: Die neonatale Sepsis ist, sowohl für Früh- als auch für Reifgeborene, einer der Hauptrisikofaktoren für Morbidität und Mortalität. Die frühe oder konnatale Sepsis (EONS) tritt innerhalb der ersten 72 Stunden nach Geburt auf, während die späte oder erworbene Sepsis (LOS) nach dieser Zeitspanne auftritt. Die Diagnosestellung der neonatalen Sepsis wird erschwert durch die unspezifische Symptomatik. Aktuelle Routinetests können neonatale Sepsis weder sicher ausschließen noch bestätigen. Die Verwendung von Inflammationsmarkern als Zusatztool in der Sepsisdiagnostik ist weit verbreitet. Interleukin-6 (IL-6) ist Teil des fetalen inflammatorischen Response-Syndroms (FIRS) und daher ein interessanter früher Marker für neonatale Sepsis.

Methoden: Unter Verwendung der PubMed Datenbank werden, zwischen 1990 und 2020 publizierte Studien, zur diagnostischen Güte von IL-6 für die Diagnose von neonataler Sepsis, identifiziert und systematisch untersucht. Diese Arbeit gibt einen Überblick über die Verwendung von IL-6, sowohl alleine als auch in Kombination mit anderen Inflammationsmarkern, für die Diagnose der frühen oder späten Sepsis des Neugeborenen. Das diagnostische Potential von IL-6 wird in Abhängigkeit des Gestationsalters, sowie von der Art der Probe und dem Zeitpunkt der Probenentnahme bewertet. Die Parameter Sensitivität, Spezifität und positiver sowie negativer Vorhersagewert werden extrahiert und eine Subgruppenanalyse durchgeführt. Die Qualität der inkludierten Studien wird mittels einer für Neugeborene mit neonataler Sepsis adaptierten STARD Checkliste bewertet.

Resultate: 34 Studien zur diagnostischen Güte von IL-6 zur Diagnostik von EONS aus dem Zeitraum 1990-2020 wurden identifiziert und hatten eine Gesamtzahl von 3508 Kindern. Der Median der Sensitivitäten lag bei 83% (Spannweite 42.1 – 100%), der Median der Spezifitäten bei 83.3% (Spannweite 43 – 100%). IL-6 erzielte eine bessere diagnostische Genauigkeit bei Frühgeborenen als in einer Gruppe mit gemischtem Gestationsalter. Sensitivität (83% versus 71%) und Spezifität (85% versus 77%) von IL-6 waren höher in Nabelschnurblut, als in peripherem Blut. Die Kombination IL-6 und CRP hatte eine vergleichbare Sensitivität zum Einzelmarker IL-6 gemessen in Nabelschnurblut (84% versus 83%), aber deutlich niedrigere Spezifität (61% versus 85%).

Konklusion: IL-6 ist ein guter diagnostischer Marker der EONS innerhalb einer Studienpopulation von Frühgeborenen, die besten Resultate wurden mit cut-off Werten

größer 30pg/mL erzielt. Die Biomarker Kombination aus IL-6 und CRP ist hoch sensitiv aber nicht sehr spezifisch. Eine Optimierung der cut-off Werte, des Zeitpunkts der Probenentnahme, sowie des Kriteriums für einen positiven Test könnten das diagnostische Potential von Biomarker Kombinationen verbessern.

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Abbreviations

CRP ..... *C reactive protein*
ECS ..... *Elective caesarean section*
EONS ..... *Early-onset neonatal sepis*
FIRS ..... *Fetal inflammatory response syndrome*
IL ..... *Interleukin*
LOS ..... *Late onset sepsis*
mRNA ..... *Messenger ribonucleic acid*
OR ..... *Odds Ratio*
PCT ..... *Procalcitonin*
pPROM ..... *Preterm premature rupture of membranes*
PROM ..... *Premature rupture of membranes*
ROC ..... *Receiver operating characteristic*
ROM ..... *Rupture of membranes*
SNAP ..... *Score for Neonatal Acute Physiology*
VLBW ..... *Very low birth weight*

1 Introduction

The work of this thesis resulted in the publication of two papers, which are included below. The first is a narrative review providing an overview on the role of inflammatory markers for the diagnosis of neonatal sepsis. It discusses the topic of neonatal sepsis, both early (EONS) and late onset neonatal sepsis (LOS), in the preterm and term infant; the diagnostic methods available; the fetal inflammatory response syndrome (FIRS); the characteristics of an ideal biomarker for diagnosis of neonatal sepsis; different inflammatory markers and their dynamics in neonatal sepsis; as well as future developments (1). Since it contains most of the original introduction of this work, this introduction has been shortened heavily. It gives, however, a more detailed analysis of the inflammatory marker IL-6 for the diagnosis of neonatal sepsis from the findings of current literature. The second paper contains the results of our meta-analysis on IL-6 diagnostic accuracy for the diagnosis of early onset neonatal sepsis (2).

Since the aim of this review is to assess the diagnostic value of IL-6 in the diagnosis of EONS and LOS under consideration of the gestational age at birth, some definitions are considered necessary. These will be used throughout the thesis, unless otherwise noted.

In this work EONS was defined as neonatal sepsis occurring within the first 72h of life, with LOS presenting after this time period, as it seems to be the most consistently used classification (3).

Term neonates are defined as infants born at 37 (37+0) weeks of gestation or later, preterm neonates accordingly as those born at an earlier gestational age. Sometimes late preterm or near term are used to describe infants born between gestational week 35 (34+0) and 36 (36+6) (4). Some studies included, focused on infants with very low birth weight (VLBW), which is defined by the World Health Organisation as a birth weight of less than 1500g (5).

Premature rupture of membranes (PROM) is defined as rupture of the membranes and leakage of amniotic fluid preceding the onset of uterine contractions and

cervical changes (6, 7). It is usually considered when delivery or onset of labour does not occur within a certain time interval after rupture of membranes (7, 8). Preterm PROM (pPROM) is the term used when PROM occurs in gestations of less than 37 completed weeks (8).

1.1 Interleukin-6

The cytokine interleukin-g (IL-6) is a particularly early marker of neonatal sepsis. It is released within 2 h after the onset of bacteraemia, peaks at approximately 6 h and finally declines over the following 24 h (9). IL-6 levels are significantly elevated up to 48 h prior to the onset of clinical sepsis (10). IL-6 is characterized by a short half-life due to binding to plasma proteins such as α2-macroglobulin, early storage in the liver or inhibition by other cytokines (11). While some investigators have found that the neonates IL-6 response is comparable to that found in adults, others have reported a diminished IL-6 production (12, 13). Stress and tissue injury have the potential to provoke an IL-6 response (14, 15). Interpretation of IL-6 levels for diagnosis of neonatal sepsis therefore might be hampered by underlying illnesses and their severity. To improve the diagnostic capacity of this early marker, combinations with later and more specific biomarkers (e.g. C reactive protein [CRP]) have been suggested (16). A relatively large sample size is required since IL-6 circulates at rather low levels (17).

1.1.1 Reference values and confounding factors

Most neonatal sepsis studies are conducted in NICUs (17, 18)l(19). Therefore control groups mostly consist of neonates with risk factors of sepsis instead of healthy neonates (14, 20, 21). Blood collection in healthy newborns is further considered unethical (22). The lack of healthy control groups, however, constitutes a problem as knowledge of reference values and normal dynamics is crucial when analysing the diagnostic value of a new potential biomarker (23). Studies of reference values have been performed, amongst others, by Chiesa et al. (24) and Barug et al. (25).

A series of confounding factors might additionally affect IL-6 levels during the early postnatal period (23). Possible confounders include neonatal age (24), length of

labour (26) and mode of delivery (25), use of epidural analgesia or vacuum extraction (25), physiologic severity and risk indices (27), and presence of clinical chorioamnionitis (28).

Chiesa et al. (24) studied upper reference limits and dynamics of IL-6 over the first 48h of life in 148 healthy babies (113 term, 35 near-term). Samples were obtained at three fixed neonatal ages (0, 24, and 48 hours after birth). Geometric mean IL-6 concentration in the healthy term babies were [1.69 pg/mL (95% CI 1.28-2.23)] at birth, [4.09 (3.13-5.33)] at 24h and [3.45 (2.70-4.43)] at 48h of life. Healthy near-term babies had corresponding IL-6 values of [10.9 (6.53-18.4)], [9.3 (6.2-14.1)] and [8.4 (5.97-11.9)] (24). Not only were IL-6 levels at all three time points higher in the near-term group than in the term group, indicating a negative association of IL-6 with gestational age, but the groups also showed significantly different kinetics of IL-6. Term babies showed significantly lower values at birth than at 24 hours, with no significant change from 24 to 48 hours, while near-term babies presented with elevated levels already at the time of birth and no significant change over the next 48 hours. The authors attribute these findings to a physiologic stress reaction which term babies seem to experience at birth, while an earlier onset, already before birth, is more likely for near-term babies (24). Several studies have linked perinatal inflammatory processes to preterm birth (29-32).

Barug et al. (25) studied the reference values of interleukin IL-6 in the cord blood of 93 healthy term neonates. Neonates showing clinical signs of infection within 72h after birth (consistent with the definition of EONS) were excluded. However, no follow-up of the sepsis status after this time point was performed. Interleukin levels were evaluated separately in neonates born via vaginal delivery (n=60) and those born via elective caesarean section (ECS) (n=33), accounting for a potential confounding effect of the mode of delivery. Higher values of IL-6 [median 3.3 (range: <2-9.5)] were found in the vaginal delivery group, versus the ECS group [2.4 (<2-12.8)] (25). These findings might be explained by the fact that contraction of skeletal muscles can enhance IL-6 levels (33, 34), as it was previously suggested for vaginal delivery (35). Claiming that the observed difference is small compared to IL-6 levels present in infected neonates, a joint reference value of <10.2 pg/mL (97.5^{th} percentile group) for healthy infants was proposed for both groups (25). In septic

infants, however, mode of delivery, spontaneously or by caesarean section did not influence IL-6 values in septic infants according to Berner et al. (36). Length of labour has been found to be associated with increased cord blood IL-6 levels in uninfected full-term neonates (26).

In a subgroup analysis Barug et al. (25) further studied the influence of epidural analgesia (n=13) and vacuum extraction (n=8) on cord blood IL-6 levels. Fever developed as side effect of epidural analgesia is difficult to distinguish from intra-uterine infection and its possible consequences for the neonate, in women during labour. IL-6 in the cord blood might serve as a means to exclude an infectious origin of fever in women who had epidural analgesia and their newborns. No significant difference was found for epidural analgesia, while vacuum extraction seemed to result in a small increase of IL-6 levels (25). However, the numbers in each group were small and larger cohorts of neonates are needed to validate these findings.

Using two objective, validated measures of neonatal illness severity, the Score for Neonatal Acute Physiology (SNAP) and its perinatal extension (SNAP-PE) (37, 38), Chiesa et al. (27) studied the differences in IL-6 levels of critically ill neonates with and without infection. They found that higher illness severity had no influence on IL-6 values in newborns with bacterial infection, but provoked a greater IL-6 response at birth in infants without infection (27).

Singh et al. showed that IL-6 concentrations in cord blood increased with clinical chorioamnionitis in neonates without evidence of infection (28).

1.1.2 Association between IL-6 and gestational age

Studies on the gestational age dependency of IL-6 levels have yielded contradicting results. While findings by Chiesa et al. indicate a negative correlation between gestational age and IL-6 levels (24), others found that IL-6 had no relation to gestational age (39) and stable levels over the course of pregnancy (6).

Whether the gestational age of an infected neonate influences its ability to produce IL-6 is still subject to research (12). In vitro preterm neonates were found to have a

lower ability to produce IL-6 in response to stimulating agents like bacterial pathogens and lipopolysaccharides, compared to term neonates (40). In the context of MIAC (Microbial invasion of the amniotic cavity), a higher fetal IL-6 response has been observed in preterm than in term gestations (41). Torbé et al. (42) report that in amniotic fluid, cytokine levels rise with gestational age, but are significantly higher in pPROM cases than in PROM at term, according to literature reviews and various study data. Andrews et al. (43) found elevated amniotic fluid IL-6 levels even in women with spontaneous labour and intact membranes for pregnancies with gestational ages < 34 weeks when compared to pregnancies 34 weeks or longer.

Hofer et al. (44) found that gestational age had no significant effect on the relationship between IL-6 and adverse neonatal outcome, but was an independent risk factor for an adverse neonatal outcome ($P < .001$).

The possibility of a gestational age-dependency of IL-6 levels in general and as response to an infection should be taken into account when evaluating the significance of IL-6 for the diagnosis of neonatal sepsis. Nevertheless many studies on IL-6 cut-off values in EONS make no difference between preterm and term neonates (11, 12, 14, 21, 45).

1.1.3 The role of IL-6 in the diagnosis of neonatal sepsis

A correlation between increased IL-6 levels and the risk for an adverse neonatal outcome has been observed by Hofer et al. (44). Odds ratios (ORs) were 7.0, 9.4 and 34.9 for IL-6 >11 pg/mL, >50 pg/mL and >500 pg/mL respectively ($P <0.001$ for all).

Due to the short half-life of IL-6 (11), elevated levels as response to sepsis are rather transient (46). In contrast to CRP levels, IL-6 levels can become normal even if infection continues (11). Sampling late in the course of sepsis leads to false-negative findings and might also explain the lower sensitivity of IL-6 observed for increasing postnatal age (11). With its short half-life IL-6 only offers a small window to detect infection, but could be useful for therapy monitoring (47).

For IL-6 an international standard for calibration of immunoassays is available from the National Institute of Biological Standards and Control of the U.K. (48). Called the WHO First International Standard for IL-6 such a standard enables comparison of results among different study groups. Details of this standard are available at https://www.nibsc.org (49).

Although often promising in first reports, newer biomarkers like IL-6, IL-8, CRP and IT ratio mostly failed to demonstrate superiority to common routine parameters when used as single markers (17). Especially in critically ill neonates, an increase in a single cytokine cannot identify bacterial infection with sufficient specificity (18). In the case of IL-6 its use as single marker is limited by its poor specificity for infection (14, 50) and a sensitivity that decreases with disease progress (11), as elaborated above.

Combinations of different markers have the ability to improve diagnostic test accuracy, but the optimal selection of markers has yet to be determined (51). Resch et al. (52) found that the combination of IL-6 with other diagnostic tests results in a consistently higher sensitivity compared to IL-6 alone. The comparison of the diagnostic value of IL-6 for sepsis in the neonate either alone or in combination with other inflammatory markers is part of this review.

If IL-6 is combined with another inflammatory marker different approaches are possible. Simultaneous measurement of several early and sensitive markers has been suggested for early identification of sepsis (18). The combination of an early and a late marker in turn might reduce the diagnostic “non-conclusive” window in sepsis diagnosis (53). An approach that might be particularly useful when sepsis diagnostic is performed at a not well defined time point i.e. at the time of sepsis suspicion.

Of six cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12, TNF-α) known to increase promptly after exposure to bacterial products, a significant increase in infants with definite or possible EONS has been shown for IL-6, IL-8 and IL-10, but not for IL-1β, IL-12 and TNF-α (18).

IL-6 and CRP represent a frequently investigated combination of an early and a late marker (18). Previous studies reported a better sensitivity and specificity for the combination than for either marker alone (18, 50). The combination with CRP is advantageous because the rise in plasma levels occurs 12 to 48 hours after the onset of infection, at a time when IL-6 levels could have fallen (11). However, at the time of sepsis evaluation CRP has a sensitivity of <50% 23 (18). A combination of IL-6 and CRP (measured at 12h of life) is effective in diagnosing EONS, but cannot guide the clinician on whether or not to start antibiotics at birth (18).

Methods for diagnosing sepsis in the neonate, those already used and those still subject to research, are manifold. Inflammatory markers have been combined with other measures in an attempt to increase diagnostic accuracy.

Clinical reliability of procalcitonin (PCT), IL-6 and IL-8 could be improved by combining them with other haematological markers and clinical signs suspicious for bacterial infection (17). Labenne et al. (18) proposed a combination of cytokines and clinical parameters to diagnose EONS. Their final score combined IL-6 and IL-8 levels (≥300 pg/mL and ≥200 pg/mL respectively) and three parameters independently associated with EONS (i.e. PROM ≥12h, maternal colonisation and mechanical ventilation at birth). Using a cut-off of ≥6.5 points their sepsis score had sensitivity of 100%, specificity of 80%, positive predictive value of 46% and negative predictive value of 100% for the diagnosis of sepsis, the AUC was 0.96.

For early identification of neonates with EONS a combination of IL-6 with perinatal data available within the first two hours of life was studied recently (50). The optimal IL-6 cut-off value determined via receiver operating characteristic (ROC) analysis was 40 ng/L. Perinatal factors considered were; PROM ≥18 hours, temperature ≥38.0 °C, CRP ≥10 mg/L, leukocytes ≥15 G/L, and an IT ratio ≥0.2 (12,13) (50). Combining IL-6 with a second factor and assuming infection if both factors were positive led to higher specificity (82.4 - 100.0%) compared to IL-6 alone (72.8%). Assuming infection in all cases where either IL-6 or a second factor was positive in turn led to an increase of sensitivity (75.0 - 92.2% vs. 75.0%) (50).

Martin et al. (54) evaluated the diagnostic value of peripheral circulatory reactive hyperemia in comparison to certain cytokines including IL-6. They found that

reactive hyperemia had equal or even higher sensitivity and specificity for the diagnosis of neonatal sepsis than cytokine levels, which could not be improved by combining it with one or more cytokines studied. A drawback of this test however is its marked inter-observer variability.

1.1.4 Timing and Site of sample collection

Ideally neonatal infection would be detected antenatal, however to date there are no non-invasive tests available to provide this early screening (42).

Postnatally, the diagnostic value of the different cytokines is limited to the time of blood collection (55). The patient can be at any point of the immunological process that constitutes the acute phase response and therefore present with very different cytokine/acute phase reactant levels (56). Furthermore, physiologic age dependent kinetics have to be considered (57), e.g. in the form of age-adjusted cut-off values (22, 46).

The period 0 to 24h of life is the critical decision time for most neonatal sepsis work-ups, especially in the era of early newborn discharge 2 (27). Failure to recognize the appropriate cut-off concentration for each time point of evaluation may confound interpretation of what constitutes a “true negative” and a “true positive” value in EONS diagnosis (27).

IL-6 is characterized by a short half-life (58) and serum levels have been shown to correlate inversely with timing of sample collection (27, 59). Elevated levels may only be detected in a certain time period after the initial stimulus (58).

Sepsis evaluation is often only performed at the time of sepsis suspicion or admission to NICU (12, 53, 55, 60). Combination of biomarkers from different phases and their simultaneous measurement may enable the detection of neonatal infections irrespective of stage (47).

Neonatal IL-6 concentrations are usually measured in either cord or peripheral blood. In comparison to peripheral blood, drawing of cord blood is less invasive, does not affect the newborn and reduces the amount of blood sampled in the

neonate, an important fact in very low birthweight infants (14, 17, 61). In cases of early neonatal sepsis that begin in utero, umbilical cord blood levels of inflammatory markers might be the ideal measure to identify those infants at risk for early neonatal sepsis (58). However, elevated cord blood IL-6 levels despite lack of evident infection are also found in newborns born to mothers with chorioamnionitis (28).

Regarding timing of sample collection cord blood is not only the earliest available neonatal sample for determination of cytokine levels, its collection also has the advantage of happening at a well-defined time point (21). Since certain cytokine levels in septic newborns drop within 24-48h after birth (36), peripheral blood samples might show normal results in infected infants. In other infants however, sampling of cord blood might be too early to show an infection, e.g. when the infection is acquired during the passage through the birth canal (21).

Absence of cytokine mRNA in neonates led to the assumption that decidual or maternal cells are the origin of the cytokine production, with cytokines reaching the fetal circulation via placental transfer (28, 62). In that case, fetal and maternal cytokine levels would well correlate; in that scenario the easier accessible maternal blood sample could be used to detect the infection status of the fetus.

IL-6 concentrations in fetal and maternal blood however did not correlate in studies by Lehrnbecher (61), Berner (36) and Messer et al. [116], while Austgulen et al. (63) found a significant correlation. Berner et al. (36) found that septic infants had higher levels of G-CSF, IL-1β, IL-6, and IL-8, but lower levels of CRP than their respective mothers. No significant difference was found for TNF-α and sICAM-1. While these findings indicate endogenous cytokine production of the neonate (36), they also show that CRP, a late-rising marker, measured in maternal blood could be of diagnostic value in the identification of septic infants.

Irrespective of the origin of elevated cytokine levels in cord blood and although maternal cytokine levels may only indicate a maternal inflammatory response, they might also be of use in the diagnosis of FIRS. It has been stated before that maternal parameters could reflect fetal infection in cases of chorioamnionitis (64).

Increased IL-6 levels have also been found in the amniotic fluid of patients with intra-amniotic infection (62). While the fetal environment undoubtly is a risk factor for the development of neonatal sepsis (58), Jung et al. (48) stress the fact that an inflammatory process in the chorioamniotic membranes in the absence of funisitis or chorionic vasculitis can only proof a maternal, but not a fetal immune response. However, various studies seem to have found a direct association between amniotic fluid interleukin elevations and EONS (42, 58).

Although assessment of various amniotic fluid mediators is highly accurate in the diagnosis of subclinical amnionitis and may be helpful in predicting early-onset neonatal infection, it requires amniocentesis which conveys certain risks like preterm labour and dissemination of infection (42, 65). Aspiration of amniotic fluid might also be difficult to perform when the amniotic fluid volume is significantly reduced, as it happens to be in cases of PROM (42). Therefore, development of prenatal non-invasive diagnostic methods for detecting intrauterine infection in patients with pPROM is highly desirable (42, 65).

Spontaneous rupture of the membranes usually occurs close to the cervix (42). In search of a non-invasive method for detecting intrauterine infection in patients with pPROM, Torbé et al. (42) hypothezised that vaginal fluid biomarker concentrations measured immediately after PROM might reflect those of the amniotic fluid. An association between proinflammatory cytokines in cervicovaginal fluid and microbial invasion of the amniotic cavity and their potential to predict neonatal infectious complications has been suggested before (42). A significant relationship has been observed for IL-6 levels in cervical fluid and amniotic fluid obtained by amniocentesis even in patients with preterm labour and intact membranes (66). However, studies like this are difficult to conduct due to the invasiveness of amniocentesis (42). Torbé et al. (42) found that the levels of all measured cytokines were significantly higher in women who subsequently gave birth to infants who developed an early onset infection. Microbial status of the cervical fluid however did not differ between women who gave birth to infants with and without infection (42).

IL-6 determination in vaginal secretion can be performed with a bedside test based on immunochromatography (67). Kayem et al. (67) found that this test predicted

neonatal infection with a sensitivity of 79%, specificity of 56%, PPV of 30%, and NPV of 92% in women with pPROM. In cases of pPROM it can be unclear whether cytokines measured in the vaginal fluid are of vaginal or amniotic origin and neonatal infection might be a result of ascending infection that developed after sample collection (42).

2 Publications

frontiers in Pediatrics

REVIEW
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Diagnosis of Neonatal Sepsis: The Role of Inflammatory Markers

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This is a narrative review on the role of biomarkers in the diagnosis of neonatal sepsis. We describe the difficulties to obtain standardized definitions in neonatal sepsis and discuss the limitations of published evidence of cut-off values and their sensitivities and specificities. Maternal risk factors influence the results of inflammatory markers as do gestational age, the time of sampling, the use of either cord blood or neonatal peripheral blood, and some non-infectious causes. Current evidence suggests that the use of promising diagnostic markers such as CD11b, CD64, IL-6, IL-8, PCT, and CRP, either alone or in combination, might enable clinicians discontinuing antibiotics confidently within 24–48 h. However, none of the current diagnostic markers is sensitive and specific enough to support the decision of withholding antibiotic treatment without considering clinical findings. It therefore seems to be justified that antibiotics are often initiated in ill term and especially preterm infants. Early markers like IL-6 and later markers like CRP are helpful in the diagnosis of neonatal sepsis considering the clinical aspect of the neonate, the gestational age, maternal risk factors and the time (age of the neonate regarding early-onset sepsis) of blood sampling.

Keywords: early onset sepsis, late onset sepsis, preterm/full term infants, inflammatory marker, interleukin-6, C-reactive protein (CRP)

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INTRODUCTION

Neonatal sepsis is still one of the leading causes of morbidity and mortality in the neonatal intensive care unit (NICU) (1). The symptoms are variable and non-specific (2). Diagnosis and treatment of neonatal sepsis remain challenging tasks even today (3). Early and efficient treatment is crucial for outcome and prognosis in neonatal sepsis cases, leading to frequent administration of empirically selected broad-spectrum antibiotics in high-risk infants (4, 5). Empirical treatment, however, increases the exposure to adverse drug effects, nosocomial complications and contributes to the development of resistant strains (6). In a cross-sectional study including 326,845 live births from 121 California hospitals with a NICU the percent of newborns with antibiotic exposure varied from 1.6 to 42.5% (7). Across hospitals, 11.4–335.7 infants received antibiotics per proven early-onset sepsis case and 2–164 infants per proven late-onset sepsis case (7). Withholding or delaying treatment in a potentially infected child, however, would be inacceptable given the rapid course and high fatality associated with neonatal sepsis (8). Biological markers that react rapidly after the onset of the inflammatory process are highly needed in the diagnosis of neonatal sepsis (9).

Neonatal sepsis is defined as either early onset sepsis (EOS) or late onset sepsis (LOS) based on whether onset of sepsis occurred before or after a certain neonatal age; and different ages have been used in the literature. According to the majority of studies EOS is defined as sepsis occurring within

the first 72 h of life, with LOS presenting after this time period. This definition is also consistent with the EOS definition by the National Institute of Child Health and Human Development and Vermont Oxford Network (10). Early studies (11), but also more recent ones (12–14) on diagnostic accuracy of IL-6, e.g., were conducted in a study population of neonatal sepsis cases without further differentiation. However, it is important to distinguish between EOS and LOS, as inclusion of patients with different neonatal ages introduces a bias due to age-related risk factors (11). The definition of abnormal biomarker values and appropriate cut-offs might significantly depend on postnatal age (11).

A large cohort study with over 108,000 very low birth weight (VLBW) infants revealed a higher mortality rate of 25.9% in case of positive blood culture compared to 11.3% for infants with negative cultures, and higher rates for EOS than for LOS (15.1% compared to 8.5%) (15). In term neonates much lower mortalities were found. In a cohort of more than 140,000 term infants mortality was 0.8% in infants with EOS vs. 0.2% in those without (16).

Early vs. Late Onset Sepsis

Neonates have only a limited repertoire of stereotypic reactions to different harmful stimuli either infectious, metabolic, respiratory, or traumatic (17) and many symptoms or signs of sepsis can be attributed to non-infectious neonatal disorders (18). EOS, in particular, presents with a different clinical course and involves other pathogens than sepsis later in life (19). Its great morbidity, mortality, and lack of early and reliable diagnostic tools make the management of EOS so challenging for the clinician (20). The incidence of culture-proven EOS in the United States is estimated to be 0.77–1 per 1,000 live births, however for infants with a body weight of $<$1,000 g, higher incidences of 26 per 1,000 are estimated (21).

EOS is mostly caused by transmission of microorganisms from the mother, happening either prenatally due to ascending colonization following the rupture of membranes or perinatal during the passage through an infected birth canal (5). Hence, causative pathogens are typically found in the maternal vaginal and fecal flora (22). Less common but also possible is an infection via the haematogenous route (19). Microorganisms prevalent in the labor or delivery room may also cause infection of the neonate (23). Simonsen et al. (21) reported Group B streptococcus (GBS) as the most common causative organism. Intra-partum antimicrobial prophylaxis however has led to a significant reduction of GBS infection rates (24). E. coli is the microorganism with the highest mortality in EOS (21).

Infants at the NICU are highly susceptible to LOS (25). A multicenter survey by Stoll et al. (26) suggested that 21% of VLBW infants who survived $>$72 h of age had at least one episode of septicaemia. In extremely low birth weight (ELBW) infants nearly two thirds experience more than one episode of suspected or culture-proven LOS during hospitalization (27). LOS has been associated with poor neurodevelopment and growth and with altered lung development (28). The most common causative organisms for nosocomial infections in neonates admitted to NICUs are Gram-positive cocci, especially coagulase-negative staphylococci (29). IL-6 has been shown to be superior to CRP in the diagnosis of late-onset neonatal sepsis due to coagulase-negative staphylococci (29). Its combination with CRP adds important information regarding withholding or stopping antibiotic therapy (29). Strunk et al. (30) hypothesized that a perinatal inflammation process might support the functional maturation of the preterm immune system, thus providing protection against LOS.

Preterm vs. Term Neonate

Consideration of gestational age in the septic infant is important for a variety of reasons. The neonatal immune system may not be fully developed (31) and cut-off values of diagnostic markers might depend on gestational age (32–35). Delayed maturation of the specific humoral and cellular immune response of neonatal B and T cells, defective activation of the complement system, and deficiencies of the myelopoetic system in the neonate have been discussed (19). Although a defective cytokine production of neonatal cells has been observed *in vitro*, *in vivo* studies did not uniformly confirm these findings (19). It has further been hypothesized that preterm infants might have a completely different immune response to sepsis than those born at term (20). Berner et al. (19) however, found that the predictive value of cord blood cytokine levels for the development of EOS does not depend on maturity and holds true for preterm infants. Yoon et al. (36) stated that the preterm fetus upon microbial invasion of the amniotic cavity is capable of mounting a systemic cytokine response, quantifiable by peripheral blood IL-6 levels. Prophylactic antibiotic treatment is often given to neonates with gestational age below 32 weeks to account for the presumed increased susceptibility to infections due to immunologic immaturity (4).

While signs of sepsis are generally subtle and unspecific in the neonate, sepsis presentation is often even more subtle in the preterm infant (3). On the other hand, preterm infants are more likely to present clinical signs like respiratory distress, apnoea, bradycardia, temperature instability, and cyanosis (3). While they could indicate sepsis, they might as well be a result of respiratory distress syndrome or prematurity itself (3, 37).

Preterm infants are more frequently born in the context of intrauterine infection (3). Infection itself has the potential to induce (preterm) labor via the secretion of pro-inflammatory cytokines secreted by the mother and/or the fetus (in response to infection) (38). Preterm infants are more likely to present with symptoms at delivery, while the majority of term neonates with EOS develops symptoms after delivery (31). These findings indicate that for preterm infants exposure to bacteria is more likely to happen *in utero*, while term neonates might be exposed to bacteria later, possibly during the passage of the birth canal (31).

Preterm labor with intact membranes and preterm premature rupture of the membranes (pPROM) are conditions frequently associated with intra-amniotic infection and inflammation (38). Neonates with pPROM have an increased risk (4–33%) of infection (39, 40). The relationship between pPROM, fetal inflammatory response syndrome (FIRS), and neonatal sepsis in preterm infants has been subject to research (39). Romero

et al. showed that in patients with pPROM, presence of FIRS led to the onset of preterm parturition (41). It is therefore not surprising that neonates with FIRS were found to have lower gestational age and lower birthweight than neonates without (22).

Morbidity and mortality of infection is particularly high in infants delivered prematurely, either due to preterm labor or preterm premature rupture of the membranes (8). The percentage of fatal neonatal infections is higher the lower the gestational age is (42) and the risk of death is 120-fold higher in preterm born population than in those delivered at term (3). Even though cord blood IL-6 levels and presence of funisitis have been found to be independent predictors of neonatal morbidity, prematurity is still considered the leading cause of perinatal morbidity and mortality (3, 6).

DIAGNOSIS OF NEONATAL SEPSIS

A positive microbiological blood culture poses the gold standard for the diagnosis of neonatal sepsis. However, much controversy exist as to the correct blood volume in neonates (43). Especially in cases of low level bacteraemia, which may account for up to two-thirds of neonatal sepsis cases, larger volumes than feasible might be needed (44). For blood samples, seeded with common neonatal pathogens, Schelonka et al. (45) demonstrated, that the sensitivity of blood cultures approaches 100% for 1 mL of inoculated blood with a bacteremia of at least 4 colony-forming units (CFU) per milliliter. Maternal antibiotic therapy under birth, while important in the prevention of neonatal sepsis, has further been discussed as possible confounder of blood culture results (46). The knowledge of these limiting factors in the group of neonates, together with high numbers of negative blood cultures in neonates with risk factors or clinical signs of EOS have led to discussions regarding the sensitivity of blood cultures in neonates (46). The reason for the high number of culture-negative cases is not clear, and diagnostic criteria used in the different publications vary substantially, so that an alternative explanation might be over-diagnosis of sepsis among non-infected infants (46). So, while sensitivity of blood culture in neonates is often quoted to be low, Cantey et al. (47) argue that there should be more focus on correctly drawn blood cultures and consequently trust in negative culture results. With results being available only after 2–3 days, time to diagnosis would be unacceptable high and hamper the use of blood cultures for early detection of sepsis (5). A recent retrospective observational study, however, showed that of 40 positive blood cultures, collected from late preterm and term infants, 39 (98%) were showing bacterial growth within 24 h. The possibility of cross-contamination or asymptomatic bacteremia might also result in a misleading or inaccurate diagnosis (44, 48).

None of the widespread laboratory markers of infection [C reactive protein (CRP), white blood cell count (WBC), absolute neutrophil count (ANC), immature to total neutrophil ratio (IT-ratio)] has enough sensitivity or specificity to detect all infected children (17, 49). Their diagnostic value might be especially limited in the early course of the disease (49). CRP, for example, is known to rise not earlier than 12–24 h after the onset of neonatal infection (2). In addition, Leucocyte count, IT- ratio, and ANC could not distinguish infected from control infants in an early sepsis evaluation (9). Hematologic tests perform poorly in differentiating between sepsis and non-infectious conditions (50). Thrombocytopenia although suggestive of systemic infection is also seen in severe lung disease due to platelet sequestration and thus not specific enough (50). The IT-ratio is determined by identifying immature neutrophil forms on a peripheral blood. Hence, its value as a diagnostic marker depends on skilful technicians. We published data on a significantly increased number of either immature granulocytes or immature myeloid information in neonates with EOS compared to controls and found their automated determination to be a useful adjunctive method in the diagnosis of EOS (51). Serial measurements of lymphocyte subsets [CD3+, CD4+, CD8+, natural killer (NK) cells, and B cells] in preterm neonates with late-onset sepsis and infection-free controls showed higher percentages of NK and B cells in the sepsis group, while those of CD3+, CD4+, and CD8+ showed no differences (52). Clinical management, especially decisions for antibiotic treatment, can't be based solely on hematologic markers (50).

Benitz et al. (44) concluded that the best-established use for laboratory markers, including hematological markers, acute-phase reactants, and inflammatory cytokines, lies in the retrospective determination that an infant was not infected, based on failure to mount an acute-phase response over the following 24–48 h. In that case, the use of these markers would offer no improvement compared to blood culture.

16S rDNA is a DNA region common to all bacteria, its detection via 16S rDNA PCR has been discussed as an alternative or addition to blood culture (24, 53, 54). PCR is not only faster it is also already considered as the gold standard in the detection of neonatal meningitis caused by herpes simplex virus (54). Al-Zahrani et al. (24) found a higher sensitivity (39 vs. 35.2%), but a lower specificity (80.5 vs. 93.5%) in comparison to blood culture for the detection of EOS in a group of proven (positive blood culture and/or positive PCR results) and clinical sepsis cases. In this study PCR was positive in 23 out of 25 blood culture positive cases (24), in another study however only 7 out of 17 cases were detected (54). Again, standardized and clinical evaluated assays for bacterial DNA detection in neonatal blood samples are lacking (24).

The options for antepartum detection of high risk fetuses are limited to historical factors, maternal clinical status, fetal behavioral assessment and the detection of amnionitis via amniocentesis (8). Risk factors for neonatal sepsis include prolonged rupture of membranes, chorioamnionitis, colonization with Group B streptococcus, prematurity, perinatal asphyxia, male gender of the fetus, foul smelling amniotic fluid, and urinary tract infection (8). However, no risk factor has consistently been able to identify a significant portion of infants with neonatal sepsis (8).

Therefore, sepsis diagnosis in neonates is typically based on a combination of maternal risk factors, hematologic indices and the judgement of the physician rather than their clinical presentation (3, 11). Early biomarkers combined with accurate

and rapid measurement methods are urgently needed for early diagnosis of sepsis and guidance of antibiotic therapy (55).

The sepsis calculator https://neonatalsepsiscalculator.kaiserpermanente.org/, a tool developed by Kaiser Permanente, provides recommendations for clinical management ranging from routine care to administration of empiric antibiotics and has been found useful for decreasing empiric antibiotic use in suspected EOS (56). A recent meta-analysis including 18 studies, with over 459,000 newborns, however, found that at initial evaluation its application assigns frequent vital signs or routine care to a substantial proportion of EOS cases, 15 and 44%, respectively (57). By 12 h of age these numbers decreased to 11.1%, and 27.8%, respectively. It is therefore important to note that the use of the EOS Calculator involves clinical monitoring beyond the initial risk classification, and clinical vigilance remains essential for all newborns (57). Since it was designed for late preterm and term neonates, the EOS calculator does not cover the high-risk population of vulnerable preterm.

Newer biomarkers investigated include acute phase proteins, cytokines and cell surface antigens (20). As mediators of the inflammatory cascade, elevated levels are likely to be observed as response to infective as well as non-infective inflammatory triggers, such as toxic processes and tissue damage (58). Ventilation may cause an inflammatory reaction in the lungs, and inflammatory markers leaving the alveolar space might appear in the systemic circulation [63]. Small sample sizes, inconsistent definitions of sepsis, heterogeneity of the study population and differences between cut-off values led to inconclusive results (20). A study (19) comparing the mRNA expression of various inflammatory markers (G-CSF, TNF, IL-1β, IL-6, and IL-8) in umbilical cord blood cells to their plasma levels in the same blood sample, found that, with the exception of TNF, mRNA expression in septic infants was not more frequently detectable than in non-septic ones. Cord blood plasma levels but not the presence of mRNA could predict EOS. Absence of mRNA could indicate that maternal cells are the origin of the cytokine production, with cytokines reaching the fetal circulation via placental transfer (59). Berner et al. (19) compared neonatal cytokine levels to the corresponding maternal blood levels. Cord blood levels of G-CSF, IL-1β, IL-6, and IL-8 were significantly higher in septic infants than in their mothers. The authors (19) therefore hypothesized that the cytokine production was triggered by an infection that occurs before birth around the time of delivery. In that case, mRNA levels might have already decreased to rarely detectable levels at the time of birth. Additionally, cell types other than mononuclear blood cells, i.e., umbilical endothelial cells, might be involved in the cytokine secretion. Production of cytokines in the gastrointestinal tract as response to ingestion of infected amiotic fluid has been discussed as a potential source of cytokines in infants with clinical sepsis syndrome and negative blood culture (3).

Systemic/Fetal Inflammatory Response Syndrome (SIRS/FIRS)

Clinical manifestations of sepsis are not limited to patients with infections, they can also be observed in patients suffering from, e.g., burns, trauma, pancreatitis, ischemia, or immune-mediated injury and result from a systemic inflammatory process (60). This phenomenon was termed "Systemic Inflammatory Response Syndrome" (SIRS) and was diagnosed if two or more of the following criteria are met in adults: Temperature $>38°C$ or $<36°C$, heart rate >90 beats/min, respiratory rate >20 breaths/min or $PaCO_2$ <32 mmHg, white blood cell (WBC) count $>12{,}000/mm^3$ or $<4{,}000/mm^3$ or $>10\%$ immature bands. In the neonatal field heart rate should be >180/min and tachypnea $>$ 60/min, WBC below $6{,}000/mm^3$ or above $30{,}000/mm^3$, and immature to total neutrophil ratio >0.2. Since vital signs, with exception of the fetal heart rate, and white blood cell counts cannot be readily determined before birth, the definition of SIRS cannot be applied to the human fetus (38).

Presence of fetal systemic inflammation akin to that observed in adult patients with SIRS was termed "Fetal Inflammatory Response Syndrome" (FIRS) and defined as an elevated concentration of fetal plasma interleukin-6 >11 pg/mL (61). Other cytokines like TNF and IL-1β were not always detected in peripheral blood with the assays available at the time (38). Its role as a major mediator of the acute phase response to infection or tissue injury further justified the choice of IL-6 as the marker of inflammation (38). Interestingly the cut-off value of 11 pg/mL obtained with ROC analysis, coincided with the two standard deviations above the mean IL-6 value in a population of 29 fetuses with subsequent normal pregnancy outcome (61). The authors found that FIRS was an independent risk factor for the occurrence of severe neonatal morbidity (61). The histopathologic counterpart of FIRS is the presence of funisitis, a polymorphonuclear leukocyte infiltration along the umbilical cord in response to infection (6, 38). Funisitis is considered to be the last stage of intra-uterine infection and, like elevated IL-6 levels, is associated with a worse neonatal outcome, including the risk of EOS (6). Despite the immaturity of the innate immune system, transcriptome analysis of patients with FIRS showed remarkable similarities between FIRS and its adult counterpart SIRS (62).

Jung et al. (38) concluded that FIRS, with the extended definition of elevation of cytokines in umbilical cord blood, presence of acute phase reactants, or severe funisitis, in preterm neonates was a risk factor for early neonatal sepsis. We found a possible association between FIRS with an adverse neonatal outcome defined as hospital mortality and/or presence of any of five morbidities including early and late onset sepsis (22). Thus, we were able to demonstrate an association between FIRS and EOS, with higher cord blood IL-6 levels in neonates with culture proven and clinical EOS. The presence of LOS however, did not show increased IL-6 levels and could not be associated with FIRS (22). In the search of an appropriate cut-off value, it is of interest that the risk for an adverse neonatal outcome correlated with the magnitude of cord blood IL-6 (22). Presence of FIRS led to a 6-fold increase in risk, values >50 pg/mL to a 9-fold and values >500 pg/mL to a 30-fold increase for an adverse neonatal outcome (22). Similar to our results (22), Strunk et al. (30) evaluating the link between histological chorioamnionitis (HCA) and neonatal sepsis showed that HCA was associated with increased risk for EOS, but seemed to reduce

the risk for LOS. The authors hypothesized that the perinatal inflammation process might enhance maturation of the neonatal immune system and therefore indirectly decreases an infant's risk for developing LOS. In contrast to these findings Jung et al. (38), pointing to the fact that LOS has been observed in infants born with FIRS after an initial period of clinical improvement, suggested that the anti-inflammatory response might play an important role in the development of LOS. Increased concentrations of anti-inflammatory cytokines like IL-10 measured in infected infants (42, 49) proof the activation of an anti-inflammatory immune response and support this theory (38).

The Ideal Biomarker

Giving thought to the requirements of an ideal diagnostic marker for neonatal bacterial infection has to fulfill, Ng (58) proposed a set of clinical and laboratory criteria. The authors (63) later extended their criteria according to a demand for more clinical information. Additional items include prediction of disease severity and provide an algorithm for antimicrobial treatment.

Considering the mortality and morbidity of neonatal sepsis, which is particularly high in preterm and VLBW infants (15), it is more important for a diagnostic test to have the highest possible level of sensitivity than the highest level of specificity (9). Ng (58) suggested a sensitivity (infected infants have a positive test) and negative predictive value (a negative test confidently rules out infection) approaching 100%, as not to withhold or delay treatment due to false negative test results (58). Specificity (the test is negative in non-infected infants) and positive predictive value (a positive test indicates true infection) should be reasonable high, i.e., above 85%, to minimize the unnecessary use of antibiotics in false positive cases (58). Such a test would not only reduce the need for extensive neonatal evaluation and empiric antibiotic treatment, but also reduce the costs related to the care of the preterm infant (37).

Tests or biomarkers with a high turnaround time are only capable to guide the discontinuation of empirical antibiotic treatment upon negative test results—a common approach in NICUs (49). An ideal marker however would be able to guide the clinician on whether to start treatment at the onset of non-specific clinical signs (63). Identification of the causative microorganism and its antibiotic susceptibility would further allow for targeted antibiotic therapy (63). Prediction of disease severity and prognosis would help the clinician at identifying those infants who are most in highly need of urgent treatment and intensive care support (63). Keeping the sample size small, i.e., a blood sample <0.5 mL, is important in neonates, especially in the group of very low birth weight infants (49, 63).

The utility of a biomarker also depends on its ability to serve as a routine diagnostic test. Specimen collection depends on clinical working hours and might be performed at different time periods in regard to sepsis onset. Hence a biomarker should be biochemically stable and maintain up- or downregulated for at least 12–24 h (63). The biomarker concentration at testing should reflect the status of the infant at the time of specimen sampling, even after transportation and storage of the sample (63). To provide the rapid turnaround time required for the early identification and appropriate management of true sepsis cases (i.e., specimen collection reporting on results <6 h), automated essays or on-demand testing in clinical laboratories might be required (63).

TABLE 1 | Criteria for an ideal biomarker or test for the diagnosis of neonatal sepsis (63).

Clinical properties

1. Such biomarkers should have a well-defined cut-off value and a sensitivity and negative predictive value approaching 100% for "ruling out" LOS (but simultaneously having high specificity and positive predictive value >85%)
2. Detect infection early (i.e., at clinical presentation)
3. Identify a specific pathogen or a category of pathogens (e.g. viral, bacterial, and fungal organisms; gram-positive organisms vs. gram-negative organisms; a specific species of pathogen)
4. Monitor disease progress and guide antimicrobial treatment (e.g. bacterial antibiotic resistance gene detection)
5. Predict the disease severity at the onset of infection (e.g. identify the type of virulent pathogen, predict DIC at the onset of disease presentation)
6. Predict prognosis (i.e., mortality)

Laboratory properties

1. Stable compound that may allow an adequate time window for specimen collection within normal working hours (i.e., sustained increase or decrease in biomarker level for at least 24 h) or easy storage of the specimen without significant decomposition of the active compound until laboratory processing
2. Quantitative determination of biomarker concentration
3. Automatic and easy method of measurement
4. Quick turnaround time (i.e., specimen collection, transport, laboratory processing time, and reporting of results to clinicians within 6 h)
5. Small volume of specimen (i.e., <0.5 mL blood)
6. Daily or on-demand availability of testing in clinical laboratories
7. Low-cost test that can be used as a routine measurement

The characteristics of the ideal biomarker according to Ng and Lam (63) are summarized in **Table 1**. Although the list was formulated in the context of LOS, its principles probably hold true for EOS diagnosis.

Once a sensitive and specific marker with a rapid reliable assay is found it must be subjected to large-scale evaluation (37). The cut-off value, i.e., the biomarker separating infected from non-infected children should be determined in a well-defined patient population using ROC analysis, thus allowing for comparison of results between NICUs (58). Most biomarker studies already rely on ROC analysis to define cut-off levels. However, within ROC analysis there are various methods to determine the ideal cut-off value. The three most common criteria for definition of a cut-off point are the following: (1) Selection of the point on the ROC curve where sensitivity and specificity are almost equal. (2) The Youden's index (sensitivity + specificity – 1) meaning the point resulting in the highest sum of sensitivity and specificity. (3) The point with the minimum distance to the upper left corner of the plot (64).

The area under the ROC curve (AUC) is an estimator of the overall accuracy of a diagnostic test (65). Biomarkers are commonly considered good or excellent if their AUCs are >0.75 or >0.9, respectively (66), the interval between 0.7 and 0.9 indicates moderate diagnostic accuracy (67). However, not all studies use these predefined definitions and moderate diagnostic

accuracy was reported for AUCs as low as 0.65 (40) and high diagnostic accuracy for AUCs of 0.751 (48). Investigators should further be aware of the need to calibrate their assays using an international standard in order to compare results among laboratories and studies (38).

Inflammatory Markers and Their Dynamics in Neonatal Sepsis

Figure 1 illustrates the inflammatory cascade showing the involved cell types and biomarkers over time [adapted from (68)]. According to their appearance and disappearance in the course of disease, the markers have been classified into early, intermediate and late markers of sepsis. **Figure 1** also shows how the level of these biomarker rise and fall during the first 48 h after onset of sepsis. Elevated in the early phase of infection are the interleukins IL-6 and−8, and CD64, ICAM, TNF, and IFN-γ, followed by the acute phase proteins PCT and CRP in the mid and late phase, respectively (69).

Cytokines and Chemokines

Upon pathogen induced activation of toll-like receptors (TLRs), proinflammatory cytokines are released from monocytes and macrophages during the early phase of the immune response starting the inflammation process (70). Cytokines have found to be promising markers of bacterial sepsis in the newborn infant (71). However, problems with invasiveness, response time, and specificity remain to be solved (72).

Tumor necrosis factor (TNF) is a proinflammatory cytokine of the first line immune response (19). It stimulates IL-6 production and acts on several types of target cells, both immune and non-immune (3). IL-6 has an inhibitory power on TNF, acting at the transcription level as well as through stimulation of the synthesis of TNF soluble receptor (3, 73). Inhibitory effects on TNF production have also been reported for prostaglandin E_2 (3). TNF levels rise rapidly, with peak blood levels at ∼1 h after the stimulus, and disappear from circulation within 3 h (9). In a study by Kashlan et al. elevated concentrations of IL-6 but not TNF were associated with infection (3). They hypothesized, that at the time of sample collection, the inflammatory response in cases of congenital sepsis had already progressed past the rapidly peaking TNF secretion (3). Leaving the resultant IL-6 elevation, as a sign that stimulation by TNF has already taken place (3).

The cytokine IL-6 is a particularly early marker of neonatal sepsis. It is released within 2 h after the onset of bacteraemia, peaks at ∼6 h and finally declines over the following 24 h (68). IL-6 levels are significantly elevated up to 48 h prior to the onset of clinical sepsis (74). IL-6 is characterized by a short half-life due to binding to plasma proteins such as α2-macroglobulin, early storage in the liver or inhibition by other cytokines (75). While some investigators have found that the neonatal IL-6 response is comparable to that found in adults, others have reported a diminished IL-6 production (2, 76). Stress and tissue injury have the potential to provoke an IL-6 response (58, 73). Interpretation of IL-6 levels for diagnosis of neonatal sepsis therefore might be hampered by underlying illnesses and their severity. To improve the diagnostic capacity of this early marker, combinations with later and more specific biomarkers (e.g., CRP) have been suggested (77). A relatively large sample size is required since IL-6 circulates at rather low levels (17). Findings in healthy infants indicate a negative correlation between gestational age and IL-6 levels (35).

The cytokine Interleukin-8 (IL-8) plays a role in the release, activation and chemotaxis of neutrophils (14). Increased IL-8 serum levels have been reported in both early- and late- onset neonatal sepsis (14). In a meta-analysis including eight studies with a total of 548 neonates pooled sensitivity and specificity of IL-8 were 78 and 84%, respectively (78). Like IL-6, IL-8 has a short half-life and its diagnostic properties have been shown to increase upon combination with CRP (28).

Acute Phase Proteins

Hepatic synthesis of the acute phase protein CRP as a response to bacterial infection takes place after stimulation by IL-6 and other proinflammatory cytokines. After synthesis, CRP in turn leads to activation of the complement system, increased phagocytosis, activation of macrophages and monocytes, and elevated production of proinflammatory cytokines (79). CRP levels begin to rise between 10 and 12 h after onset of infection, and peak at 48 h (33, 79). A relatively long serum half-life of 24–48 h has been reported for CRP (33). Due to the delayed rise of CRP levels as a response to infection CRP has an unacceptable low sensitivity within the first 24 h, i.e., for the early diagnosis of neonatal sepsis (33, 80). It further shows a non-specific physiological increase in the first 3 days of life, probably due to the stress of delivery and other non-infectious perinatal and maternal factors, hampering its use as a sepsis marker during this time period (81). Levels of up to 20 mg/L at 48 h after delivery have been reported in healthy neonates (82). Performance of serial measurements and combination of CRP with earlier markers such as CD64, interleukins or procalcitonin have the potential to improve the diagnostic accuracy in the early phases of sepsis in both EOS and LOS (33, 81). Beyond its use as diagnostic marker, CRP is particularly useful in monitoring the response to treatment and is used to guide the antibiotic therapy in neonatal sepsis (33). Benitz et al. (44) demonstrated that a persistence of normal CRP levels under antibiotic treatment strongly correlates with the absence of infection. Serial measurements are the most accurate and reliable in the diagnosis of bacterial infection of the neonate and are recommended within a time window of 24–48 h after onset of infection (21, 33, 83). Established by Mathers and Pohlandt (83), the most commonly used cut-off value for CRP during the first days of life continues to be 10 mg/L (33). By use of ROC analysis we demonstrated that CRP could play a role in the early diagnosis of neonatal sepsis if cut-off values were lowered (80). Perrone et al. (82) performing a study on CRP levels in healthy neonates stated that a static cut-off level is unable to reflect the physiological kinetics of CRP, and proposed the use of different cut-off levels adapted to gestational age, postnatal age and mode of delivery. This was confirmed by our group (32, 33) demonstrating that preterm infants had lower CRP values compared to term infants. CRP values increased by 0.405 mg/L for every 1 week increase in gestational age (32, 33). Raised CRP levels are not specific for bacterial infection, and might also appear in conditions as asphyxia, shock, intraventricular

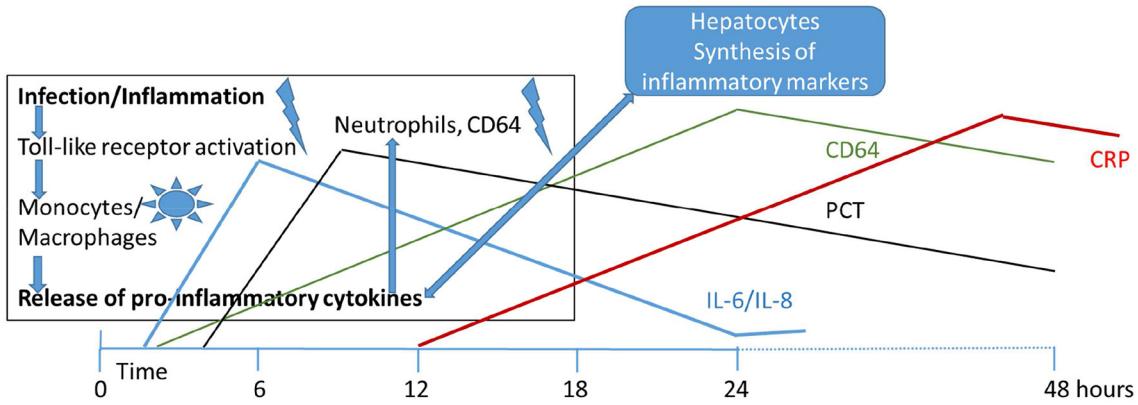

FIGURE 1 | Initiation of infectious/inflammatory processes and release of inflammatory markers over the first 48 h of life in early onset neonatal sepsis. IL, interleukin; PCT, procalcitonin; CRP, C-reactive protein; CD64, cluster of differentiation (neutrophil surface expression).

hemorrhage, surgery, and meconium aspiration (84). For viral infections only slight elevations of CRP levels (<5 mg/L) have been reported (85, 86). Non-infectious inflammatory processes, such as PROM, meconium-stained amniotic fluid and prolonged labor, however, also caused significant elevations of CRP (82). Advantages of CRP as sepsis marker include its broad availability, simplicity, speed, and low cost (82). CRP refers to high sensitive CRP (hsCRP) when high-sensitivity assay techniques are used to measure concentrations as low as 0.01 mg/L (87).

Procalcitonin (PCT), the prohormone of calcitonin, is mainly produced by monocytes and hepatocytes and shows a significant elevation during infections in neonates, children and adults (88). Elevated PCT levels as a response to infection can be detected within 6 h after its onset, peak at 18–24 h and remain elevated up to 48 h (the half-life of PCT in peripheral blood is ∼24 h) (88). Hence, PCT classifies as an early to intermediate-rising biomarker. PCT, like CRP, shows a physiological increase after birth, limiting its diagnostic value in the first 2–4 days of life (81). Studies evaluating the potential of PCT as an early marker for neonatal sepsis (89–91) have found that within the first 48 h of life elevated PCT levels were present even in uninfected or healthy neonates. However, the rise in PCT levels is much more marked in bacterial and fungal, but not viral, infections (92). Reference values and age related 95th percentile nomograms for the first days of life exist for healthy term and preterm infants (89, 92, 93) and have served as basis for the use of age-adjusted cut-off values (5, 94). In children and neonates after 72 h of age, PCT values <0.5 ng/ml seem to be normal; increases to 0.5–2 ng/ml seem to be related to non-infectious inflammation, viral or focal bacterial infections and increases above 2–2.5 ng/ml, seem to be related to bacterial or fungal systemic infections (95). In a recent multicentre, randomized controlled trial (NeoPIns) Stocker et al. (96) evaluated the potential of PCT to guide antibiotic treatment in infants with suspected EOS. They found that, within a population with low incidence of culture-proven infection, discontinuation of treatment based on PCT resulted in no adverse outcomes and duration of antibiotic therapy was significantly reduced (96). In a meta-analysis assessing the diagnostic potential of PCT in neonatal sepsis, the diagnostic accuracy seemed to be higher in neonates with LOS, than in those with EOS (5, 97). However, fewer data were available for LOS. Statistical heterogeneity and differences in the definitions used for neonatal sepsis additionally had to be taken into account. Advantages of PCT include its wide diagnostic window (88), its specificity to bacterial infections (98) and its quick reduction in response to adequate therapy (99). PCT also proved to play a role in SIRS and increased levels of PCT were associated with increased severity of disease and increased rates of mortality in adults (100).

Synthesis of the acute phase protein Serum amyloid A (SAA) is regulated by the pro-inflammatory cytokines IL-6 and TNF and takes place mainly in the liver, but also happens in smooth muscle cells, macrophages, adipocytes, and endothelial cells (81). The effect of SAA is mainly anti-inflammatory. It reduces the production of prostaglandin E_2 and the oxidative respiration of neutrophils, counteracts the pyrogenic effect of a number of cytokines, inhibits platelet activation, negatively controls the production of antibodies, and induces the secretion of collagenase by fibroblasts (101). Free SAA has been found to possess cytokine-like properties which induce chemotaxis of neutrophils, granulocytes, and T-lymphocytes (102). Differences to the acute phase protein CRP include an earlier and sharper rise in the acute phase response, which occurs not only in bacterial but also in viral infections (101). A study set out to define normal SAA levels in healthy individuals and reported median SAA levels of 0.758 mg/L (range: 0.758–3.000) for cord blood and 1.516 (0.758–10.580) for 35 neonates in each group (103). During neonatal sepsis a 1,000-fold increase in the serum concentration of SAA has been reported (104), and elevated levels have been found in early- as well as in late onset sepsis (105–107). In a meta-analysis, consisting of a total of nine studies, diagnostic accuracy of SAA in EOS and LOS (measured 8–96 h after onset of infection) was found to be moderate and comparable to those of CRP and PCT (108). However, findings were again limited by heterogeneity of study population groups and cut-off levels. Other studies reported improved sensitivity relative to CRP at

the point of clinical suspicion (106, 107). An advantage of SAA is the availability of an automated and rapid test, however the number of studies about the SAA test in neonatal sepsis is limited (108, 109).

Cell Surface Molecules

The Fcγ receptor 1 alpha chain, known as Cluster of differentiation 64 (CD64) is an immunoglobulin binding receptor found on the surface of leukocytes and showing high affinity to IgG immunoglobulin (110). In bacterial infection stimulation by proinflammatory cytokines IFN-γ and TNF and granulocyte colony stimulating factor (G-CSF) leads to an upregulation of CD64 expression (5–10-fold in comparison with baseline levels) (111, 112). The increase of CD64 is associated with the intensity of the triggering cytokine release (113). CD64 in turn induced enhanced antigen presentation, and facilitated phagocytosis and intracellular killing of opsonized microbes (110, 114).

In healthy subjects, antigen-presenting cells (monocytes, macrophages, and dendritic cells) express CD64, while neutrophils show only very low levels of CD64 expression (115). The later however rises by ongoing infection, converting it into an interesting sepsis marker. The neutrophil CD64 expression, often referred to as nCD64, is measured either alone or as a ratio to the monocyte CD64, which did not increase significantly (116). During bacterial infection nCD64 expression was markedly increased in all age groups, but, interestingly, higher levels were found in healthy preterm and term neonates when compared with healthy adults (115). Increased nCD64 expression was detected within 1–6 h after bacterial invasion and levels remained elevated for >24 h while viral infections were not associated with an increased nCD64 expression (112). Promising results were published for CD64 as a diagnostic marker in both EOS and LOS, but study heterogeneity led to a wide range of sensitivity and specificity, respectively (81). Increased CD64 expression has been considered as an independent risk factor for LOS, which has to be taken into account when its diagnostic value is evaluated in LOS (117). Advantages of CD64 as sepsis marker include the wide diagnostic window, the very small amount of blood needed (±50 μL of whole blood), easy handling and rapid turnaround time being <1 h (81, 118, 119). Serial measurements of CD64 were suggested for guiding antibiotic therapy in neonates (120). CD64 quantitative flow cytometric analysis could be developed into a routine clinical test with high comparability and reproducibility across different laboratories (121). However, to this date there is a lack of consistent cut-off values for CD64 and further research is needed to define the optimal cut-off value and time point of measurement, before CD64 expression testing could be incorporated in the clinical practice (122, 123).

DISCUSSION

Despite the promising results reported by many studies, most diagnostic markers fail to meet the criteria required for clinical practice. Cost, availability of specimens at the appropriate time, complexity of the assay methods, laboratory turnover time, reliability of the tests, and experience of the attending clinicians are all important factors in determining the accuracy of a diagnostic marker for clinical use (58).

Assays of chemokines and cytokines, as well as tests measuring the expression of cell surface antigens are expensive (58). However, 10% of all deliveries are preterm births and most of them have complete blood cell counts and intravenous access. If further sepsis evaluation is added, blood cultures (costs at $65 per set) and lumbar punctures (costs at $162 per procedure, $129 for cultures, cell count, protein, and glucose studies) add to an economic impact of current infant sepsis evaluation that is impressive (43). While the costs for 3 days of empiric antibiotic therapy with ampicillin and gentamicin are moderate between $12 and $15, high facility costs are generated due to prolonged NICU stays (37). Hence, effective testing strategies that enable a reduction of extended sepsis evaluation and empirical treatment would result in tremendous cost savings (37).

The usefulness of new inflammatory markers depends on the laboratory turnover time. Performing analyses in batches, or if possible (CD64) postponing sample collection until the next working morning, hampers their use as "early warning markers" (58). Finally, a more recent meta-analysis revealed low sensitivities and specificities and, thus, concluded to use it cautiously in the diagnosis of neonatal sepsis (also poorer performance in preterm than tem infants) (124). To satisfy the rapid turnover time required clinically a trained technician needs to be available at all times, something not practicable in most institutions (58). Therefore, *ad-hoc* measurement will only become cost effective if assay methods become automated (58).

As with blood culture the limiting factor for measuring combinations of cytokines is the large volume of serum required. Conventional enzyme-linked immunosorbent assay techniques require about 100–250 μL to quantify one protein (49). Something not feasible, especially in very low birth weight (VLBW) infants (49). Multiplex systems based on flow cytometry allow for the simultaneous quantitative measurement of several biomarkers with only a minimal volume of blood (49). For example only 50 μL of plasma are required for the measurement of six cytokines, or 50 μL of whole blood for each surface antigen measurement (49, 58). However, these are not the typical platforms used to quantitate analyses in clinical medicine (38) and intention-to-treat studies are required to examine their potential for reducing unnecessary antibiotic treatments (49). Ng (58) saw the use of the flow cytometric technology in the identification of cytokines or cell surface markers most suitable for clinical use.

A recent analysis (125) of 480 episodes of suspected LOS in 208 preterm infants below 32 weeks of gestational age showed that serum IL-6 and PCT levels (hazard ratios 2.28 and 2.91, respectively), but not CRP (hazard ratio 1.16), were associated with sepsis severity and mortality risk. These findings might select neonates at risk who will need more intensive monitoring and therapy (125). Thus, inflammatory markers might serve as prognostic parameter for severity of neonatal sepsis and mortality. A further study by Kurul et al. (126) on LOS showed that application of a decision tree incorporating inflammatory

markers (IL-6, PCT, CRP) reached a diagnostic accuracy of nearly 88%.

Serial measurement of infection markers are thought to improve the diagnostic sensitivity of these tests. The combination of an early sensitive marker with a late specific one might enhance the diagnostic accuracy of the markers (58). Serial physical examination has been suggested as an alternative or additional tool to serial determination of inflammatory markers (127). For us this is quasi a "conditio sine qua non" in the treatment of seriously ill septic neonates. Current evidence suggests that the use of promising diagnostic markers like CD11b, CD64, IL-6, IL-8, PCT, and CRP, either alone or in combination, might be helpful when considering to discontinue antibiotics at 24–48 h of onset of the suspected infection process. In case of an infant that remains clinically well waiting for the definitive microbiological results would not be any more necessary. However, none of the current diagnostic markers are sensitive and specific enough to support the decision to withhold antibiotic treatment (58).

Even if these issues are resolved the crucial factor seems to be the difficulty to define the clinical usefulness of infection markers from the findings of the current literature (58). Mehr et al. (71) stated, back in 2000, that the heterogeneous methods of laboratory measurement and the wide variations in data analysis including cut-off values and the resulting differences in reported conclusions precluded the possibility of performing a meaningful meta-analysis. Problems that remain an issue even today (20). Reliable cut-off values are either lacking or there is an abundance of different cut-offs proposed for the same marker, both renders a potential diagnostic test wearisome to apply clinically (58). For a marker to serve as a routine diagnostic tool, high comparability and reproducibility across different laboratories is required (58).

Future Aspects and Conclusion

There are few reports on the use of proteomic analysis from patients with sepsis, and the results have not been validated by well-established techniques (128). A recent review identified nearly 200 proteins in response to sepsis by proteomic analysis of septic blood, of whom some might serve as sepsis markers (129). The problem with proteomic analyses that identify specific proteins and peptides by random sampling of disease and control plasma from different patients and from different clinical settings is the retrospective interpretation of findings (130). For early identification of septic neonates we are faced with the same old problems of each biomarker as demonstrated in this review. In addition, this is further true for metabolomics in septic patients (131).

The clinical usefulness of pediatric heart rate in predicting clinical deterioration (e.g., pediatric sepsis) is limited by the lack of consensus among warning systems, consensus-based guidelines, and evidence-based studies as to what constitutes abnormal heart rate in the pediatric age group (132). The authors of this recent review concluded that current studies on heart rate variability do not adequately discriminate children with sepsis from those without. Maybe only in combination with biomarkers a better interpretation of the findings is possible or vice versa.

In conclusion, despite lots of promising inflammatory markers, the clinical ability to discriminate between infected and uninfected neonates remains to be a challenge, and antibiotics are often initiated in ill term and especially preterm infants. Hence, for the early diagnosis IL-6 (from cord blood or peripheral neonatal blood) and later repetitive measurements of CRP seem to be helpful in the diagnosis of neonatal sepsis considering the clinical aspect of the neonate, its gestational age, maternal risk factors, and the time of sampling.

AUTHOR CONTRIBUTIONS

JE and BR were responsible for the writing of the manuscript. ER for the literature search, table, and figure. BR finally edited the last version of the manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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SYSTEMATIC REVIEW
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Reliability of Interleukin-6 Alone and in Combination for Diagnosis of Early Onset Neonatal Sepsis: Systematic Review

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Neonatal sepsis is a major cause of morbidity and mortality in both preterm and term infants. Early-onset neonatal sepsis (EONS) presents within the first 72 h of life. Diagnosis is difficult as signs and symptoms are non-specific, and inflammatory markers are widely used to confirm or rule out neonatal sepsis. Interleukin-6 (IL-6) is part of the fetal inflammatory response syndrome (FIRS) and therefore an interesting early marker for neonatal sepsis. The main objective for this review was to assess the diagnostic potential of IL-6, alone and in combination, for diagnosis of early neonatal sepsis (EONS) in term and preterm infants, in cord and peripheral blood, and in dependence of timing of sample collection. IL-6 diagnostic accuracy studies for diagnosing EONS published between 1990 and 2020 were retrieved using the PubMed database. We included 31 out of 204 articles evaluating the potential of IL-6 for the diagnosis of EONS in a study population of newborns with culture-proven and/or clinically suspected sepsis. We excluded articles dealing with neonatal bacterial infections other than sepsis and biomarkers other than inflammatory markers, those written in languages other than English or German, studies that did not distinguish between EONS and late-onset sepsis, and animal and *in vitro* studies. Full-text articles were checked for other relevant studies according to the PRISMA criteria. We identified 31 studies on IL-6 diagnostic accuracy for EONS diagnosis between 1990 and 2020 including a total of 3,276 infants. Sensitivity and specificity were reported, and subgroup analysis was performed. A STARD checklist adapted for neonates with neonatal sepsis was used for quality assessment. The range of IL-6 sensitivity and specificity in neonatal samples was 42.1–100% and 43–100%; the median values were 83 and 83.3%, respectively. IL-6 accuracy was better in preterm infants than in mixed-study populations. Early sample collection at the time of sepsis suspicion had the highest sensitivity when compared to other time points. Cord blood IL-6 had higher diagnostic value compared to peripheral blood. The biomarker combination of IL-6 and CRP was found to be highly sensitive, but poorly specific. Limitations of this review include use of only one database and inclusion of a heterogeneous group of studies and a small number of studies looking at biomarker combinations; a strength of this review is its focus on early-onset sepsis, since type of

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sepsis was identified as a significant source of heterogeneity in IL-6 diagnostic accuracy studies. We concluded that IL-6 has a good performance as an early diagnostic marker of EONS within a study population of preterm infants, with best results for cord blood IL-6 using cutoff values above 30 pg/ml.

Keywords: interleukin-6 (IL-6), early onset neonatal sepsis, diagnostic accuracy, sensitivity and specificity, meta-analysis

INTRODUCTION

Neonatal sepsis is still one of the leading causes of morbidity and mortality in the neonatal intensive care unit (NICU) (1). The symptoms are variable and non-specific (2). Diagnosis and treatment of neonatal sepsis remain challenging (3). Early and efficient treatment is crucial for a good neonatal outcome and prognosis in neonatal sepsis cases often necessitating empirically selected broad-spectrum antibiotics in high-risk infants (4, 5). Empirical treatment, however, increases the exposure to adverse drug effects and nosocomial complications and contributes to the development of resistant strains (6). For the United States, it has been shown that for every neonate with proven bacterial sepsis, 11–30 infants with negative sepsis status receive antibiotics (7). Withholding or delaying treatment in a potentially infected child, however, would be inacceptable given the rapid course and high fatality associated with neonatal sepsis (8). Biological markers that react rapidly after the onset of the inflammatory process are greatly needed in the diagnosis of neonatal sepsis (9).

Interleukin-6 (IL-6) is characterized by a short half-life due to binding to plasma proteins such as α2-macroglobulin, early storage in the liver, or inhibition by other cytokines (10). The cytokine IL-6 is a particularly early marker of neonatal sepsis. It is released within 2 h after the onset of bacteremia, peaks at approximately 6 h, and finally declines over the following 24 h (11). IL-6 levels are significantly elevated up to 48 h prior to the onset of clinical sepsis (12). While some investigators have found that the neonatal IL-6 response is comparable to that found in adults, others have reported a diminished IL-6 production (2, 13). Stress and tissue injury have the potential to provoke an IL-6 response (14, 15). Interpretation of IL-6 levels for diagnosis of neonatal sepsis might therefore be hampered by underlying illnesses and their severity. To improve the diagnostic capacity of this early marker, combinations with later and more specific biomarkers (e.g., CRP) have been suggested (16). A relatively large sample size is required since IL-6 circulates at rather low levels (17).

Chiesa et al. (18) studied the upper reference limits and dynamics of IL-6 over the first 48 h of life in 148 healthy babies (113 term, 35 near-term). Samples were obtained at three fixed neonatal ages (0, 24, and 48 h after birth). The geometric mean IL-6 concentrations in the healthy term babies were 1.69 at birth, 4.09 at 24 h, and 3.45 pg/ml at 48 h of life. Healthy near-term babies had corresponding IL-6 values of 10.9, 9.3, and 8.4 pg/ml (18).

IL-6 is one of the most studied cytokines in sepsis; its circulating levels rise rapidly in response to infection and are closely associated with sepsis prognosis and mortality in adults (2, 5). For septic neonates, divergent results have been published, ranging from diminished IL-6 production in term and even more pronounced in preterm infants to IL-6 concentrations comparable to that found in adults (2). In neonates, IL-6 is an early and highly sensitive marker (4, 19). Interestingly, IL-6 levels in cord blood correlate well with neonatal hematologic indices used to evaluate EONS (8). However, the specificity of IL-6 is often low (4), and increased IL-6 values were also found in infants with non-infectious conditions limiting its use in the differentiation of neonates having infections or not (14).

A crucial factor for the implementation of inflammatory markers for neonatal sepsis diagnosis seems to be the difficulty to formulate a definitive opinion on their clinical usefulness from the findings of current literature (15). Small sample sizes, inconsistent definitions of sepsis, heterogeneity of the study population, and differences between cutoff values led to inconclusive results in diagnostic accuracy studies (20). Mehr et al. (21) stated, back in 2000, that the heterogeneous methods of laboratory measurement and the wide variations in data analysis and in reporting results precluded the possibility of performing a meaningful meta-analysis—problems that remain an issue even today (20). Either reliable cutoff values are lacking or there is an abundance of different cutoffs proposed for the same marker, both rendering a potential diagnostic test wearisome to apply clinically (15). The aim of the study was to determine the actual role of IL-6 alone or in combination for the diagnosis of EONS by means of a meta-analysis including studies from 1990 to 2020, to identify factors that possibly affect the diagnostic potential of IL-6 and investigate them by means of a subgroup analysis.

METHODS

Studies eligible for review inclusion were retrieved using the PubMed database including diagnostic accuracy studies of IL-6 in neonates published between 1990 and 2020. The combined search term used was (Interleukin-6 OR IL-6) AND (neonatal sepsis OR neonatal infection OR sepsis) AND (early-onset sepsis OR EOS OR EONS). No PubMed filters or language restrictions were used.

Reviewer (JE) conducted the database search and identified potential studies by screening titles and abstracts. For inclusion, the following criteria had to be fulfilled on an abstract level: the study population consisted of newborns, the subjects presented with culture-proven and/or clinically suspected sepsis, and the article evaluated the potential of IL-6 (alone or in combination with other inflammatory markers) for the diagnosis of early-onset neonatal sepsis. Excluded were articles dealing with other neonatal bacterial infections, those written in languages other

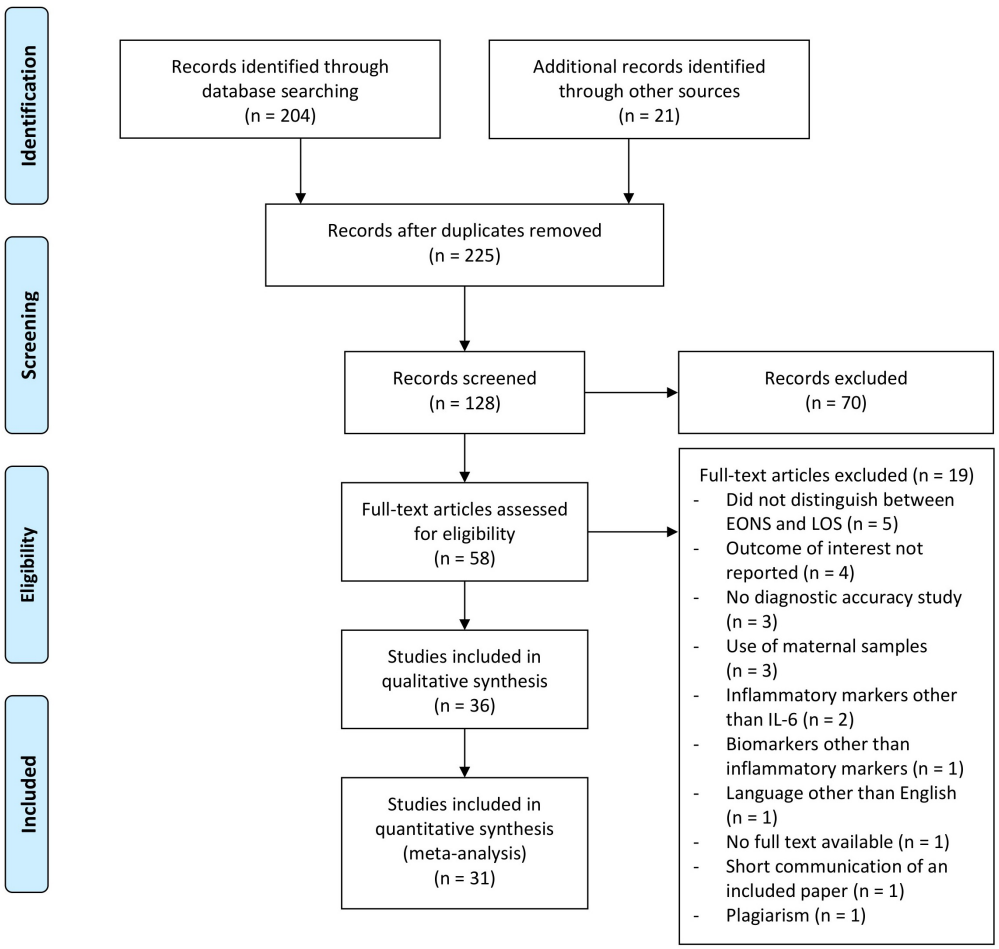

FIGURE 1 | Flowchart of the study selection process for diagnostic accuracy of interleukin-6 in early onset neonatal sepsis between 1990 and 2020.

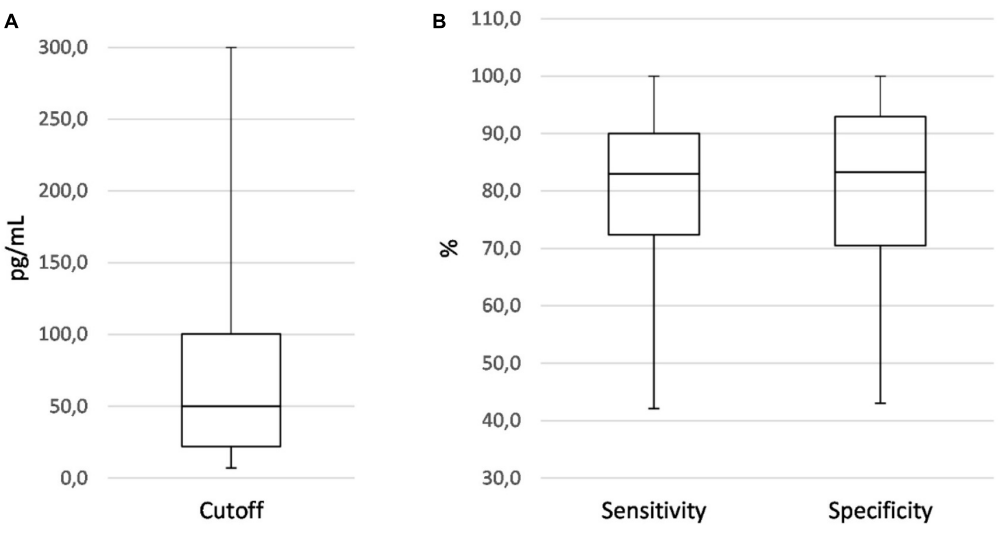

FIGURE 2 | Boxplots showing the distribution of IL-6 cutoff **(A)**, sensitivity, and specificity values **(B)** of all diagnostic accuracy studies on EONS using neonatal samples.

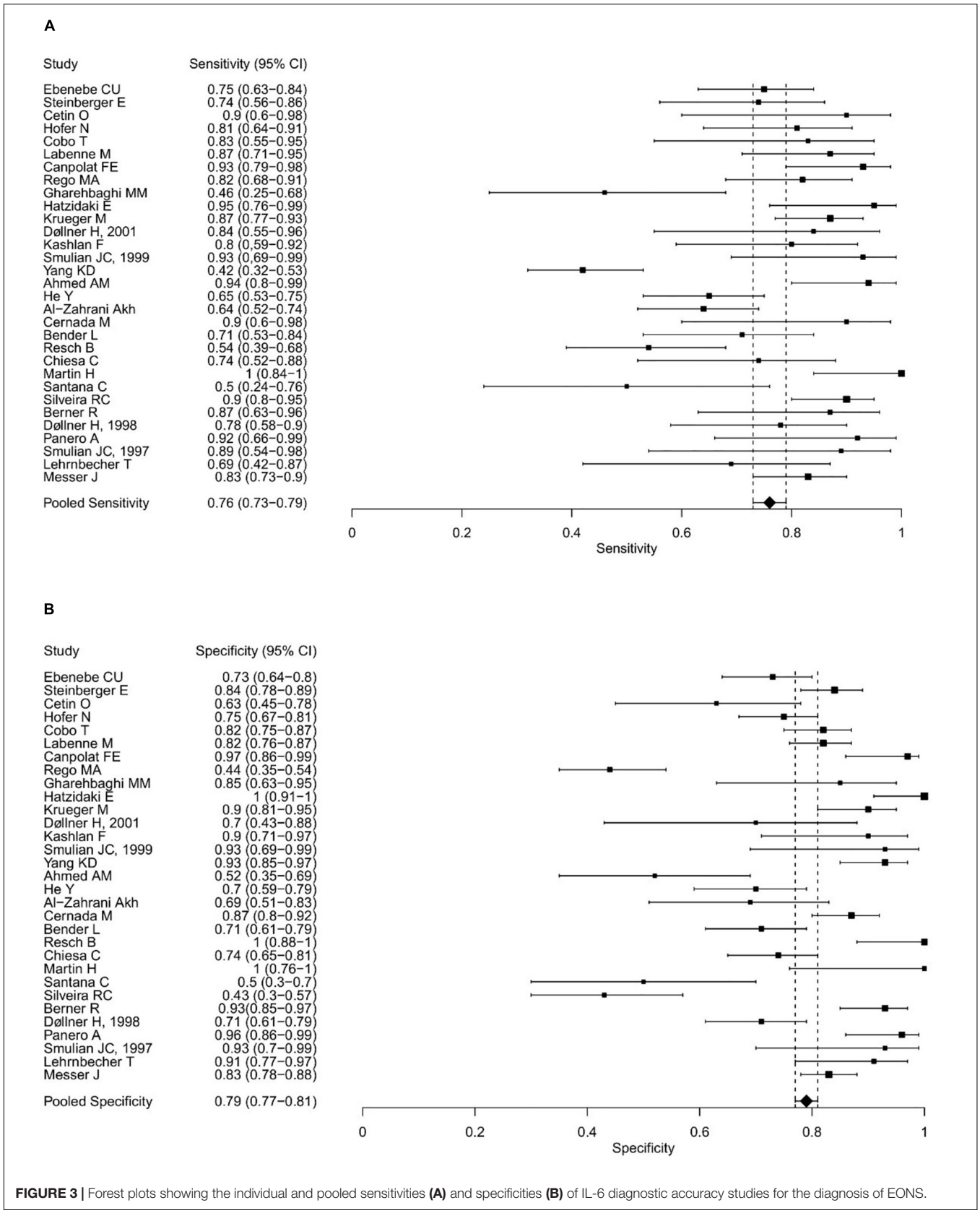

FIGURE 3 | Forest plots showing the individual and pooled sensitivities **(A)** and specificities **(B)** of IL-6 diagnostic accuracy studies for the diagnosis of EONS.

than English or German, studies that did not distinguish between EONS and late-onset sepsis, animal and *in vitro* studies, and studies analyzing biomarkers other than inflammatory markers. Subsequently, full-text articles of shortlisted studies were assessed for eligibility (JE). Reference lists of obtained articles and relevant review articles were hand searched for other relevant studies according to the PRISMA criteria (see **Figure 1**). In cases of doubt, study eligibility was resolved with input from an independent reviewer (BR). Relevant data from the eligible studies were extracted by reviewer JE using a standardized data collection form. Data extraction included the following: first author, country, year of publication, sepsis definition, and number and specific characteristics of the newborns in the septic and non-septic groups (summarized under recruitment). Further data included reference standards employed, sample studied, time of sample collection, whether IL-6 was used alone or in combination with another inflammatory marker, and finally the test method used. The analyses were based on previously published studies. Therefore, no patient consent, ethical approval, and institutional review board were required.

Reviewer JE assessed the quality of reporting of the included studies using a data extraction form based on the STARD checklist and adapted for neonates with neonatal sepsis by Chiesa et al. (22). The key domains—descriptions of participant recruitment, reference standard and index test, and study population—are evaluated simply answering yes, if the item is present, or no if not (22).

Statistics included evaluation of studies regarding sensitivity and specificity of IL-6, the cutoff value, and the area under the curve (AUC) of IL-6 for sepsis diagnosis. The sensitivity indicates the percentage of patients with sepsis diagnosis who had a plasma cytokine level above the given cutoff, specifically the percentage of patients not meeting the criteria of sepsis who had cytokine levels below the cutoff value. Box-plot diagrams were used to show the distribution of cutoff levels used and sensitivity and specificity values reported, while forest plots show sensitivity and specificity values of the individual studies as well as pooled sensitivity and specificity. The 95% confidence intervals were calculated using Wilson's method (23).

By performing extensive literature research, we identified factors that have been shown to influence IL-6 levels in neonates, namely, gestational age, type of sample, time of sample collection, and choice of cutoff value. Since those factors represent possible causes of heterogeneity in diagnostic accuracy studies of IL-6, we subsequently researched their influence on study results by subgroup analysis within our meta-analysis. Pooled sensitivities and specificities were calculated by grouping studies, coinciding with influencing factors together, and treating their infants as one big study population. To investigate the influence of gestational age, subgroups were formed by grouping all preterm infants and all infants from mixed (preterm and term) study populations together. To further evaluate the influence of a low vs. a high cutoff value, these subgroups were again divided by the median cutoff level present within the respective subgroup. The influence of type of sample was investigated by forming subgroups of cord versus peripheral blood samples. The peripheral blood sample subgroup was further divided into those studies reporting sample collection times earlier than 48 h of life (e.g., $\leq$ 6 h, $\leq$ 12 h) and studies allowing larger time intervals for sample collection (up to 1 week). Meta-analysis on a certain biomarker combinations was performed if at least 3 studies studied this combination.

RESULTS

As shown in **Figure 1**, 204 records on IL-6 as a marker of EONS were identified from the PubMed database search and 21 additional records were identified by screening the reference lists of included articles and relevant review articles (22, 24–27). Following exclusion of studies by titles, 128 abstracts were screened. The full text of 58 articles was assessed, and finally 31 studies including 3,276 infants were eligible for meta-analysis.

Most studies agreed on the definition of EONS, as sepsis occurring before 72 h of life (4–6, 8, 19, 20, 28–32). Other definitions included sepsis $\leq$ 48 h (n = 5) (33–37), sepsis $\leq$ 1 week (n = 1) (38), sepsis $\leq$ 5 days (n = 1) (9), sepsis $\leq$ 4 days of life (n = 1) (39), and sepsis within the first days of life (n = 1) (40). Eleven studies did not specify their definition (2, 3, 7, 10, 14, 17, 41–45).

The range of IL-6 sensitivities and specificities was 42.1–100% and 43–100%, respectively; the median values were 83 and 83.3% (see boxplots in **Figure 2**). Pooled sensitivity was 76% (95% CI: 73–79%), pooled specificity 79% (77–81%) (see forest plots in **Figure 3**). The data extracted from the included IL-6 diagnostic accuracy studies is summarized in **Table 1** for preterm infants, in **Table 2** for mixed study populations, and in **Table 3** for IL-6 in combination with other inflammatory markers.

Results of the subgroup analysis are shown in **Table 4**. Sensitivity was higher in the group of preterm infants (83 vs. 73%), while specificity did not vary among study populations (both 82%). Within the preterm group, a cutoff value $\geq$30 provided slightly improved sensitivity (84 vs. 80%) and specificity (82 vs. 81%) compared to a lower cutoff value. Within the group of preterm and term infants, a cutoff $\geq$80 pg/ml led to a drastic increase in specificity (90% vs. 71%) but not sensitivity (both 73%). The sensitivity and specificity in umbilical cord blood was higher than in neonatal peripheral blood, 83 vs. 71% and 85 vs. 77%, respectively. Six (7, 9, 33, 35, 36, 45) of the 12 studies (2, 5, 7, 9, 19, 31, 33, 35, 36, 38, 43, 45) using peripheral blood as sample reported sample collection times earlier than 48 h of life (e.g., $\leq$6 h, $\leq$12 h), and studies allowing larger time intervals for sample collection (e.g., <4 days) were grouped under <1 week. Early sample collection ($\leq$48 h) improved the sensitivity of a peripheral blood sample (80 vs. 71% in the overall group), but not the specificity (both 77%). The lowest sensitivity was observed for late sampling (<1 week) of peripheral blood (64%); specificity, however, was not worse than in the early sampling group (77%).

Six studies reported results from IL-6 combined with either CRP (n = 3) or procalcitonin (PCT, n = 2) or tumor necrosis factor alpha (TNF-α, n = 1) (2, 4, 9, 17, 32, 42). In the three studies analyzing a combination of IL-6 and CRP, cutoff values ranged from 36 to 100 pg/ml and 10 to 60 mg/l, respectively, sensitivities between 75 and 100%, and specificities between 37 and 74% (2, 4, 42). The pooled sensitivity was comparable to that of cord blood

TABLE 1 | Characteristics of IL-6 accuracy studies for diagnosing EONS in the preterm infant.

Author, year, country, (reference)	EONS definition	Recruitment	Reference standard in infected neonates	Reference standard in control neonates	Sample studied, time of sample collection	Test	IL-6 cutoff (pg/mL)	Sensitivity, % (95% CI)	Specificity, % (95% CI)	AUC (95% CI)	PPV (%)	NPV (%)
Ebenebe et al., Germany, (4)	≤72 h	182 preterm infants with a birth weight <2,000 g: 67 infected, 115 uninfected	(1) Positive blood culture or (2) CRP ≥ 5 mg/l and ≥ 3 clinical signs	Gestational age and birth-weight matched neonates that did not fulfill criteria of EONS	Neonatal blood, 0 h (PNA)	Electrochemi luminescence assay	40	75	72.8	0.804	14	98
Steinberger et al., Austria, (17)	NS	218 NICU preterm infants with risk factors for EONS: 30 infected, 188 uninfected	(1) Positive blood culture or (2) ≥3 categories of clinical signs or (3) ≥1 categories of clinical signs, and ≥2 laboratory abnormalities (CRP, WBC, I:T ratio)	NA	Cord blood, 0 h (PNA)	ELISA	15.85 (ROC, Youden)	73.7 (51.2–88.2)	84.2 (75.8–90)	0.812 (0.675–0.948)	46.7 (30.2–63.9)	94.4 (87.6–97.6)
Cetin et al., Turkey, (41)	NS	40 preterm infants born to mothers with pPROM: 10 infected, 30 uninfected	Positive blood or gastric washing culture and/or clinical findings	NA	Cord blood, 0 h (PNA)	ELISA	11 (ROC, NS)	90 (55–98)	63.3 (43–80)	0.767 (0.608–0.926)	45	95
Hofer et al., Austria, (28)	≤72 h	176 preterm infants at risk of bacterial infection: 32 EONS, 144 other	(1) Positive bacterial culture from umbilical cord blood, peripheral blood, or CSF or (2) negative culture, but ≥3 categories of clinical sepsis signs, with either ≥1 maternal risk factors or ≥2 abnormal laboratory markers (CRP, WBC, I:T ratio)	NA	Cord blood (UV), 0 h (PNA)	ELISA	11.1 (ROC, Youden)	81	75	0.795 (0.695–0.896)	NA	NA
Cobo et al., Czech Republic, (6)	≤72 h	176 preterm infants born to mothers with PPROM: 12 infected, 164 uninfected	(1) Positive blood culture or (2) clinical signs and ≥2 abnormal hematological laboratory results (WBC, PC, I:T ratio)	NA	Cord blood, 0 h (PNA)	ELISA	38 (ROC, NS)	83	82	0.908 (0.846–0.971)	30	98.1

(Continued)

TABLE 1 | (Continued)

Author, year, country, (reference)	EONS definition	Recruitment	Reference standard in infected neonates	Reference standard in control neonates	Sample studied, time of sample collection	Test	IL-6 cutoff (pg/mL)	Sensitivity, % (95% CI)	Specificity, % (95% CI)	AUC (95% CI)	PPV (%)	NPV (%)
Labenne et al., France, (7)	NS	213 NICU preterm infants with a presumptive diagnosis of EONS: 31 infected, 182 uninfected	(1) Positive culture of blood or CSF, and clinical signs or (2) clinical signs, CRP >1 mg/dl, positive superficial or placental cultures, and no alternative diagnosis	(1) Positive superficial culture without abnormal CRP or (2) CRP > 1 mg/dl and an alternative diagnosis or (3) neither positive culture nor abnormal CRP	Venous blood, at sepsis evaluation (≤6 h PNA)	Cytometric bead array (a multiplexed system)	300 (ROC, minimizing number of misclassified episodes)	87.1 (71.1–94.9)	82	0.895 (0.837–0.953)	NA	97.3
Canpolat et al., Turkey, (40)	Within the first days of life	74 preterm infants born to mothers with pPROM: 32 infected, 42 uninfected	(1) Positive blood culture and clinical signs and/or abnormal laboratory findings or (2) negative blood culture, but clinical and/or laboratory findings	Negative blood culture and no clinical or laboratory findings	Cord blood (UV), 0 h (PNA)	ELISA	7.6 (ROC, NS)	93	96.7	NA	NA	NA
Rego et al., Brazil, (42)	NS	144 NICU preterm infants presenting RDS during the first 24 h of life: 44 infected, 100 infected	In addition to RDS, (1) ≥2 categories of clinical signs, or clinical chorioamnionitis, and positive blood or CSF culture or (2) ≥ 2 categories of clinical sepsis, or clinical chorioamnionitis, and a hematologic sepsis score > 3 or 3) radiographic evidence of pneumonia and a hematologic sepsis score > 3	No clinical signs and a hematologic sepsis score <3	Peripheral blood, 0 h (from suspicion of sepsis)	Chemilu minescence immunoassay	36 (ROC, maximum sensitivity and specificity >50%)	82 (67–93)	44 (33–55)	0.72 (0.62–0.83)	40 (29–51)	85 (71–94)
Gharehbaghi et al., Iran, (29)	≤72 h	45 NICU preterm infants born to mothers with PROM: 17 infected, 18 uninfected	(1) Positive blood culture or (2) negative blood culture, but ≥3 clinical signs of sepsis associated with laboratory findings (WBC, platelet count, I:T ratio)	NA	Cord blood, 0 h (PNA)	ELISA	20	46	85	NA	88	39
Hatzidaki et al., Greece, (43)	NS	58 preterm neonates born to mothers with pPROM: 20 infected, 38 uninfected	(1) Positive blood culture within 4 days of life or (2) ≥3 categories of clinical signs and ≥2 abnormal laboratory findings	NA	Cord blood, 0 h (PNA)	ELISA	108.5 (ROC, NS)	95	100	NA	100	97.4
					Neonatal blood, on day 4 (PNA)	ELISA	55 (ROC, NS)	90	97.4	NA	94.7	94.9

(Continued)

TABLE 1 | (Continued)

Author, year, country, (reference)	EONS definition	Recruitment	Reference standard in infected neonates	Reference standard in control neonates	Sample studied, time of sample collection	Test	IL-6 cutoff (pg/mL)	Sensitivity, % (95% CI)	Specificity, % (95% CI)	AUC (95% CI)	PPV (%)	NPV (%)
Krueger et al., Germany, (34)	≤48 h	Of the 136 infants, 77 were preterm: 40 infected, 37 uninfected	(1) Clinical signs and positive blood culture or (2) clinical signs and abnormal laboratory results (CRP, I:T ratio), biological fluids positive for bacteria, or signs of inflammation in placenta	Non-infectious clinical conditions	Cord blood, 0 h (PNA)	Fully automated chemil uminescence immunoassay	80 (ROC, ULC)	96	94	NA	NA	NA
Døllner et al., Norway, (44)	NS	24 NICU preterm infants: 11 infected, 13 uninfected	(1) Clinical signs, and a positive blood culture or (2) ≥3 categories of clinical signs, and CRP ≥ 3 mg/dl or (3) radiographic evidence of pneumonia, respiratory signs or symptoms, and CRP ≥ 3 mg/dl	Clinical conditions apparently non-infectious	Cord blood, 0 h (PNA)	ELISA (Quantikine)	33	84	70	0.86 (0.66–0.96)	NA + L9:O12	NA
Kashlan et al., United States, (3)	NS	43 NICU singleton, very preterm infants (≤32 weeks GA): 21 infected, 22 uninfected	(1) Positive culture of blood and/or CSF or (2) ≥3 maternal/neonatal indicators for infection (risk factors, clinical signs, abnormal hematological findings)	Negative blood culture and <3 maternal/neonatal indicators for infection	Cord blood (UV), 0 h (PNA)	Enzyme-linked immunoassay (Endogen)	100 (ROC, NS)	80	90	NA	89	83
Smulian et al., United States, (30)	≤72 h	28 preterm infants with either spontaneous preterm labor or PPROM: 14 infected, 14 uninfected	(1) Autopsy or positive CSF or blood culture or (2) clinical signs and ≥2 laboratory abnormalities (WBC, I:T ratio, PC, abnormal CSF analysis)	NA	Cord blood (UV), 0 h (PNA)	ELISA (Quantikine)	25 (ROC, ULC)	92.9	92.9	NA	92.9	92.9

NS, bot specified; NA, bot available to NS, not specified; NA, not available; UV, umbilical vein; UA, umbilical artery; PNA, postnatal age; NICU, neonatal intensive care unit; CSF, cerebrospinal fluid; CRP, C-reactive protein; WBC, white blood count; PC, platelet count; ABC, absolute band count; EONS, early-onset neonatal sepsis; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value; GA, gestational age.

IL-6 (3, 6, 8, 14, 17, 20, 28–30, 34, 35, 37, 40, 41, 43, 44, 46) as a single measure (84 vs. 83%), but the pooled specificity was markedly lower (61 vs. 85%).

The assessment of the overall quality of the included studies based on the STARD checklist is summarized in **Table 5**. All 31 articles included in the meta-analysis were studies of diagnostic accuracy of IL-6, and most resulted from single perinatal centers. Enrollment of patients was based on maternal and prenatal risk factors in seven studies (6, 20, 28, 29, 40, 41, 43) and on clinical signs in further nine studies (4, 7, 17, 19, 31, 32, 34, 38, 44). Three studies included neonates having already been diagnosed with sepsis (4, 38, 44). Almost all of the included IL-6 diagnostic accuracy studies used different reference standards to diagnose EONS and verify index test results leading to differential verification bias. Only three studies used a composite reference standard to exclude sepsis (7, 14, 46). In ten studies, CRP was used as comparator of the index test but also formed part of the reference standard (2, 4, 5, 10, 14, 17, 19, 38, 44, 45). Clinical and demographic data were reported in 22 studies (2–7, 9, 14, 19, 30–36, 38, 40–42, 44, 46). Most studies analyzed birth weight and gestational age as indicators of illness severity, but three studies relied on measures of illness severity that are more objective (7, 35, 42). About a third of the studies (11/31) stated how many neonates failed to undergo the index tests and/or the reference standard (6, 10, 29, 33–35, 38, 40, 42, 44, 45). In the majority of studies, cutoff values were defined *post hoc*. At least 7 studies (6, 8, 10, 19, 35, 41, 46) reported the number, training, and expertise of the persons executing and reading the index test and the reference standard, and 11 studies (2, 6, 8, 10, 19, 29, 31, 35, 42, 43, 46) provided information about masking. Measures of statistical uncertainty (i.e., 95% confidence intervals) and handling of indeterminate results, missing responses, and outliers of index tests were among the least commonly reported items from the STARD checklist (only 8 studies). Fourteen studies provided information regarding methods for calculating IL-6 test reproducibility (3, 7, 9, 10, 14, 20, 30, 34, 35, 41–44).

DISCUSSION

Our meta-analysis including 31 studies with 3,276 infants resulted in a pooled sensitivity of IL-6 of 76% and pooled specificity of 79%. A recent review including 31 studies with 1,448 infants reported a global sensitivity and specificity of 82% (77–86%) and 88% (83–92%), respectively (47). Only 6 studies (7, 9, 32, 36, 40, 45) of this review (47) were also included in our review, partly due to the missing differentiation between early- and late-onset sepsis in their meta-analysis. Differences to our meta-analysis further included selection process, missing differentiation of cord vs. peripheral blood, preterm vs. term infants, influence of pPROM, time of sampling, and combination with other markers. Finally, we included twice as many infants.

We used subgroup analysis to analyze the influence of gestational age in the study population, the cutoff level used, the type of sample, and the time of sample collection. Three studies had to be excluded from the subgroup analysis (32, 33, 42). Reasons were a modification of the cutoff criteria, e.g., to favor a high specificity, so as not to introduce a bias in the subgroup analysis (32, 42), or data provided for illustrative purposes only [Panero et al. (33), data for postnatal day 1]. Some groups provided data for different scenarios, e.g., analyzing different samples or varying the cutoff level. For the subgroup analysis of preterm vs. preterm and term infants, each study was only included once, so as not to introduce the same study population multiple times within the same subgroup analysis. This was done by choosing the scenario yielding the best results, or, if subgroups were analyzed, the one including the whole study population. One study did not specify whether they used cord or peripheral blood and was excluded from the subgroup analysis regarding the type of sample (4). Subgroup analysis showed that sensitivity of IL-6 was higher in the group of preterm infants compared to the mixed group of preterm and term infants (83 vs. 73%), while specificity did not vary among study populations (both 82%). Even though a wide cutoff range of 11–300 pg/ml resulted from the included publications, we found that a cutoff value $\geq$30 provided only slightly improved sensitivity (84 vs. 80%) and specificity (82 vs. 81%) compared to a lower cutoff value in a group of preterm infants. These findings are in agreement with Qiu et al. (25). In the group of preterm and term infants, however, a cutoff of $\geq$80 pg/ml led to a drastic increase in specificity (90 vs. 71%) but not sensitivity (both 73%). In general, sensitivity and specificity values were found to vary greatly among different studies even for the same cutoff value, thus suggesting a different source of heterogeneity. We found a higher pooled sensitivity (83 vs. 71%) and specificity (85 vs. 77%) for umbilical cord blood compared to peripheral blood samples. In contrast, Qiu et al. (25) found a higher sensitivity and specificity of IL-6 in peripheral blood within a population of pPROM infants. Our results revealed improved sensitivity (80 vs. 71%) and constant specificity (77%) of early sampling within the first 48 h from peripheral blood.

EONS was more frequently observed in infants with pPROM than in premature infants with intact membranes (38 vs. 10%, $p = 0.001$) (29). Infants with pPROM had increased cord blood IL-6 levels, which were significantly higher in neonates who developed EONS and thus had a higher predictive value than clinical signs of chorioamnionitis (29). Another group found that cord blood IL-6 but not funisitis in women complicated with pPROM was an independent predictor for the occurrence of EONS (6). A meta-analysis investigating IL-6 as a diagnostic tool after pPROM included nine studies and reported a pooled sensitivity of 85% and specificity of 88% (25). The cutoff values ranged between 7.8 and 108.5 pg/ml correlating with sensitivities between 46 and 95% and specificities between 63.3 and 100%. Two-thirds of studies reported cord blood IL-6 values and mixed populations of preterm and term infants.

Six studies included analyzed biomarker combinations (2, 4, 9, 17, 32, 42). Messer et al. (10) stated that IL-6 appeared to be an ideal marker before the age of 12 h and in combination with CRP, thereafter leading to a sensitivity of 100%. However, this was hardly surprising since an elevated CRP level was one of the classification criteria. The combination of IL-6 > 36 pg/ml (0 h) and/or CRP > 60 mg/l (24 h) was able to increase sensitivity (93 vs. 82%). However, the specificity remained low

TABLE 2 | Characteristics of IL-6 accuracy studies for diagnosing EONS in a mixed population of preterm and term infants.

Author, year, country, (reference)	EONS definition	Recruitment	Reference standard in infected neonates	Reference standard in control neonates	Sample studied, time of sample collection	Test	IL-6 cutoff (pg/mL)	Sensitivity, % (95% CI)	Specificity, % (95% CI)	AUC (95% CI)	PPV, %	NPV, %
Yang et al., China, (5)	≤72 h	152 preterm (>34 weeks) and term infants at risk for EONS: 76 infected, 76 uninfected	(1) Positive blood or CSF culture or (2) ≥3 categories of clinical signs	Negative blood culture and <3 categories of clinical signs	Venous blood, ≤72 h (PNA)	MILLIPLEX Map Human Th17 Magnetic Bead Panel and Sepsis Panel (Millipore)	153	42.1	93.4	0.704 (0.622–0.786)	84.6	61.4
Ahmed et al., Egypt, (31)	≤72 h	60 NICU preterm and term infants: 30 high suspicion of EONS, 30 matched controls	Clinical findings supporting the suspicion of neonatal sepsis	Age- and weight-matched neonates without the criteria of sepsis suspicion	Venous blood, ≤72 h (PNA)	ELISA	24 (ROC, Youden)	94.4	52.4	0.751 (0.623–0.854)	45.9	95.7
He et al., China, (19)	≤72 h	151 preterm (>34 weeks) and term infants with suspected EONS: 68 infected, 83 uninfected	(1) Positive blood or CSF culture and any abnormal finding or (2) negative culture results but ≥3 abnormal findings	Negative culture results and <3 abnormal findings	Venous blood, ≤72 h (PNA)	MILLIPLEX Map Human Th17 Magnetic Bead Panel and Sepsis Panel (Millipore)	75.43	64.71	69.88	0.706 (0.626–0.777)	63.77	70.74
Al-Zahrani et al., Saudi Arabia, (38)	<1 week	100 NICU preterm and term infants with suspected sepsis: 71 infected, 29 uninfected	(1) Positive blood culture and/or positive PCR results for bacterial 16S rDNA or (2) negative blood culture and PCR, but clinical signs of sepsis and positive sepsis screen.	Neonates suspected of having sepsis with negative blood culture, PCR and sepsis screen	Blood sample, ≤24 h (after NICU admission), <1 week (PNA)	ELISA	60	63.6	69	NA	75.6	55.5
Cernada et al., Spain, (20)	≤72 h	128 preterm and term infants with prenatal risk factors for EONS (77% asymptomatic at birth): 10 infected, 118 uninfected	(1) Positive blood culture and clinical sings or (2) ≥3 categories of clinical signs	NA	Cord blood, 0 h (PNA)	Chemiluminescence enzyme immunoassay in solid phase	255.87 (ROC, NS)	90	87.4	0.88 (0.7–1.06) (*sic*)	37.5	99
Bender et al., Denmark, (32)	≤72 h	123 NICU preterm and term infants with at least 1 clinical sign suggesting EONS: 29 infected, 94 uninfected	(1) Positive blood culture or (2) clinical signs and CRP > 5 mg/dl	(1) Clinical signs and CRP ≤ 5 mg/dl and antibiotic therapy for 3 days or (2) clinical signs, but no antibiotic therapy	Peripheral blood, 0 h (after suspicion of sepsis)	Flow cytometry (LUMINEX)	250 (ROC, specificity ∼95%)	59 (41–75)	94 (87–97)	0.77	76	88
							12 (ROC, sensitivity ∼ specificity)	71	71	0.77	43	89
Resch et al., Austria, (45)	NS	68 NICU preterm and term infants with suspected sepsis: 41 infected, 27 uninfected	(1) Positive blood culture or (2) ≥3 categories of clinical signs, positive sepsis screen and/or risk factors, and antibiotic therapy ≥7 days	Negative blood culture, negative sepsis screen, and antibiotic therapy ≤3 days	Venous or arterial blood, ≤12 h (PNA)	ELISA	≥10 (ROC, NS)	71 (56–82)	67 (48–81)	NA	76	60

(Continued)

TABLE 2 | (Continued)

Author, year, country, (reference)	EONS definition	Recruitment	Reference standard in infected neonates	Reference standard in control neonates	Sample studied, time of sample collection	Test	IL-6 cutoff (pg/mL)	Sensitivity, % (95% CI)	Specificity, % (95% CI)	AUC (95% CI)	PPV, %	NPV, %
							≥60 (ROC, Youden)	54 (39–68)	100 (88–100)	NA	100	59
							≥150 (ROC, NS)	46 (32–61)	100 (88–100)	NA	100	55
Chiesa et al., Italy, (35)	≤48 h	134 NICU preterm and term infants: 19 infected, 115 uninfected	(1) Positive blood culture and clinical signs or (2) ≥3 clinical signs prompting ≥5 days of antibiotic therapy, and historical and clinical risk factors for EONS	Symptomatic infants who had negative body fluid cultures, and were apparently well within 24–48 h and received antibiotic treatment ≤3 days	Cord blood, 0 h (PNA)	ELISA	200 (ROC, Youden)	74 (51–88)	89 (82–93)	NA	NA	NA
					Peripheral blood, 24 h (PNA)		30 (ROC, Youden)	63 (41–81)	71 (62–78)	NA	NA	NA
					Peripheral blood, 48 h (PNA)		20 (ROC, Youden)	53 (32–73)	70 (63–79)	NA	NA	NA
Martin et al., Sweden, (36)	≤48 h	32 NICU preterm and term infants with suspected sepsis: 20 infected, 12 uninfected	(1) Positive blood or CSF culture or (2) abnormal CRP, WBC and ≥1 category of clinical signs (i.e., oliguria, metabolic acidosis, or hypoxemia)	Clinical conditions apparently non-infectious	Peripheral blood, at admission, ≤48 h (PNA)	Chemiluminescence immunoassay	160 (ROC, Youden)	100	70	NA	67	100
Krueger et al., Germany, (34)	≤48 h	136 preterm and term infants: 68 infected, 68 uninfected	(1) Clinical signs and positive blood culture or (2) clinical signs and abnormal laboratory results (CRP, I:T ratio), biological fluids positive for bacteria, or signs of inflammation in placenta	Non-infectious clinical conditions	Cord blood, 0 h (PNA)	Fully automated chemiluminescence immunoassay	80 (ROC, ULC)	87	90	NA	NA	NA
Santana et al., Spain, (14)	NS	31 preterm and term infants: 10 infected, 11 uninfected, 10 healthy controls	≥2 categories of clinical signs, ≥1 abnormal laboratory findings, and positive blood culture	(1) Clinical conditions apparently non-infectious or (2) GA-matched neonates with normal postnatal course through the first month of life	Cord blood, 0 h (PNA)	Chemiluminescence enzymoimmunoassay in the solid phase	100.8 (ROC, NS)	50	87	∼0.5	31	66

(Continued)

TABLE 2 | (Continued)

Author, year, country, (reference)	EONS definition	Recruitment	Reference standard in infected neonates	Reference standard in control neonates	Sample studied, time of sample collection	Test	IL-6 cutoff (pg/mL)	Sensitivity, % (95% CI)	Specificity, % (95% CI)	AUC (95% CI)	PPV, %	NPV, %
Silveira and Procianoy, Brazil, (9)	≤5 days	117NICU infants with suspected sepsis: 66 infected, 51 uninfected	(1) Positive blood and/or CSF culture and ≥3 categories of clinical sepsis or (2) negative cultures and ≥3 categories of clinical sepsis	PROM, but no complete criteria for clinical sepsis, no antibiotic treatment up to discharge from hospital, no hospital readmission (<1 month)	Peripheral blood, 0 h (after suspicion of sepsis), 82.9% at ≤24 h (PNA)	Quantitative sandwich enzyme immunoassay technique (Quantikine)	32 (ROC, NS)	90	43	NA	67.4	78.6
Berner et al., Germany, (46)	≤4 days	136 preterm and term infants, cord blood samples available in 93 infants: 16 infected, 43 uninfected, 35 healthy controls	(1) Positive blood culture or (2) ≥3 categories of clinical signs or laboratory markers	(1) Clinical suspicion but neither positive culture, nor ≥3 categories of clinical signs or	Cord blood, 0 h (PNA)	Double-sandwich enzyme immunoassay (Quantikine)	100 (NA)	87	93	NA	76	97
Døllner et al., Norway, (2)	NS	113 NICU preterm and term infants: 24 infected, 89 uninfected	(1) Positive blood/CSF culture and clinical signs for sepsis/meningitis or (2) negative blood culture, ≥3 categories of clinical signs and abnormal laboratory results (CRP, I:T ratio) or (3) negative blood culture, respiratory symptoms, X-ray consistent with pneumonia, and abnormal laboratory results	Initially suspected of having an infection (not confirmed)	Peripheral blood, at NICU admission or on the next day, >92% <4 days (PNA)	IL-6–dependent mouse hybridoma cell line B13.29 (clone B9), as described by Ng [(15) cite]	20 (NA)	78	71	NA	40	93
							50 (NA)	61	76	NA	38	89
Panero et al., Italy, (33)	≤48 h	60 NICU preterm and term infants: 13 infected, 47 uninfected	Positive blood culture and clinical signs of sepsis	Infants with various types of distress and non-specific abnormal clinical signs who were well within 48–72 h	Venous blood, ≤ 24 h (PNA)	Solid-phase sandwich enzyme-amplified sensitivity immunoassay (Medgenix)	70 (ROC, NS)	69	36	NA	23	81
					Venous blood, ≤24 h (PNA)		200 (ROC, NS)	38	70	NA	26	80
					Venous blood, 24–48 h (PNA)		50 (ROC, NS)	92	96	NA	86	98

(Continued)

TABLE 2 | (Continued)

Author, year, country, (reference)	EONS definition	Recruitment	Reference standard in infected neonates	Reference standard in control neonates	Sample studied, time of sample collection	Test	IL-6 cutoff (pg/mL)	Sensitivity, % (95% CI)	Specificity, % (95% CI)	AUC (95% CI)	PPV, %	NPV, %
Smulian et al., United States, (8)	≤72 h	23 preterm and term infants with suspected EONS: 8 infected, 15 uninfected	(1) Positive blood or CSF culture or (2) clinical signs and ≥laboratory abnormalities (WBC, I:T ratio, PC, ABC, or abnormal spinal tap)	NA	Cord blood (UA), 0 h (PNA)	ELISA (Quantikine)	7 (NA)	88.5	66.6	NA	58.8	91
					Cord blood (UV), 0 h (PNA)	ELISA (Quantikine)	7 (NA)	88.5	93.3	NA	88.5	93.3
Lehrnbecher et al., Germany, (37)	≤48 h	46 NICU preterm and term infants: 13 infected, 33 uninfected	(1) Positive blood culture and ≥3 categories of clinical signs or (2) negative blood culture, ≥3 categories of clinical signs and ≥2 abnormal laboratory results in the first 48 h of life	NA	Cord blood, 0 h (PNA)	Enzyme immunoassay (Dianova-Immunotech)	150 (ROC, NS)	69	91	NA	NA	NA
Messer et al., France, (10)	NS	288 NICU/obstetric unit preterm and term infants: 71 infected (36 infected or probably infected, 35 possibly infected, 217 uninfected	(1) Positive blood and/or CSF culture, clinical signs, and abnormal laboratory results (CRP, WBC) or (2) Negative culture results but ≥3 categories of clinical signs and abnormal laboratory results or (3) negative culture results, <3 categories of clinical signs, abnormal laboratory results that could have another reason, neither exclusion nor confirmation of sepsis possible	Neither clinical nor biological signs of infection	Cord or peripheral blood, NA	ELISA (Hoffmann-La Roche)	100 (ROC, ULC)	83.3	90.3	NA	NA	NA
		Of the 288 infants, 220 were inborn: 39 infected (18 infected or probably infected, 21 possibly infected), 181 uninfected			Cord or peripheral blood, ≤1 h (PNA)			100	92.3	NA	58.8	97
		Of the 288 infants, 254 were sampled within the first 12 h of life: NA			Cord or peripheral blood, ≤12 h (PNA)			100	89	NA	NA	NA

NA, not available; NS, not specified; UV, umbilical vein; UA, umbilical artery; PNA, postnatal age; NICU, neonatal intensive care unit; CSF, cerebrospinal fluid; CRP, C-reactive protein; WBC, white blood count; PC, platelet count; ABC, absolute band count; EONS, early-onset neonatal sepsis; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value; GA, gestational age.

TABLE 3 | Characteristics of IL-6 accuracy studies for diagnosing EONS using biomarker combinations.

Author, Year, Country, (Reference)	EONS definition	Recruitment	Reference standard in infected neonates	Reference standard in control neonates	Sample studied, time of sample collection	Test	Biomarker combination	Criterion for positive test	Cutoffs: IL-6 (pg/mL), CRP (mg/L), PCT (ng/mL), TNF-α (pg/mL)	Sensitivity (95% CI), %	Specificity (95% CI), %	AUC	PPV, %	NPV, %
Ebenebe et al., Germany, (4)	≤72 h	1,202 preterm infants with a birth weight < 2,000 g: 67 infected, 115 uninfected	(1) Positive blood culture or (2) CRP ≥ 5 mg/l and ≥ 3 clinical signs	Gestational age and birth-weight matched neonates that did not fulfill criteria of EONS	IL-6: neonatal blood, 0 h (PNA) and maternal blood (CRP), <24 h (before delivery)	IL-6: electrochemiluminescence assay, CRP: particle enhanced immune-nephelometry	IL-6 + CRP	and	IL-6: 40, CRP: 10	49.0	82.4	NA	14.1	96.5
							IL-6 + CRP	Either/or		90.2	43.1	NA	8.6	98.7
					Neonatal blood 0 h (PNA)	IL-6: electrochemiluminescence assay, CRP: particle enhanced immune-nephelometry	IL-6 + CRP	and	IL-6: 40, CRP: 10	23.4	100	NA	100.0	96.8
							IL-6 + CRP	either/or		75	71.7		13.6	98
Steinberger et al., Austria, (17)	NS	218 NICU preterm infants with risk factors for EONS: 30 infected, 188 uninfected	(1) Positive blood culture or (2) ≥3 categories of clinical signs or (3) ≥1 categories of clinical signs, and ≥2 laboratory abnormalities (CRP, WBC, I:T ratio)	NA	Cord blood	IL-6: ELISA, PCT: LUMItest procalcitonin kit	IL-6 + PCT	and	IL-6: 10, PCT: 0.5	58.8	99.0	0.850 (0.731–0.968)	NA	NA
							IL-6 + PCT	Either/or	IL-6: 15.85, PCT: 0.235 (ROC, Youden)	91.7 (71.2–99.0)	77.1 (67.4–85.0)	0.915 (0.822–1.000)	42.1 (26.3–59.2)	98.7 (92.8–99.8)
Rego et al., Brazil, (42)	NS	144 NICU preterm infants (130 VLBW) presenting RDS during the first 24 h of life: 44 infected, 100 infected	In addition to RDS, (1) ≥ 2 categories of clinical signs, or a hematologic or clinical chorioamnionitis, and positive blood or CSF culture or (2) ≥2 categories of clinical sepsis, or clinical chorioamnionitis, and a hematologic sepsis score >3 or 3) radiographic evidence of pneumonia and a hematologic sepsis score >3	No clinical signs and a hematologic sepsis score <3	Peripheral blood	Chemilumines cence immunoassay system	IL-6 + CRP	and/or	IL-6: 36, CRP: 60 (ROC, maximum sensitivity and specificity >50%)	93 (80–98)	37 (27–48)	NA	41 (31–51)	92 (78–98)

(Continued)

TABLE 3 | (Continued)

Author, Year, Country, (Reference)	EONS definition	Recruitment	Reference standard in infected neonates	Reference standard in control neonates	Sample studied, time of sample collection	Test	Biomarker combination	Criterion for positive test	Cutoffs: IL-6 (pg/mL), CRP (mg/L), PCT (ng/mL), TNF-α (pg/mL)	Sensitivity (95% CI), %	Specificity (95% CI), %	AUC	PPV, %	NPV, %
Bender et al., Denmark, (32)	EONS (=72 h)	123 NICU preterm and term infants with at least 1 clinical sign suggesting EONS: 29 infected, 94 uninfected	(1) Positive blood culture or (2) clinical signs and CRP > 5 mg/dl	(1) Clinical signs and CRP ≤ 5 mg/dL and antibiotic therapy for 3 days or (2) clinical signs, but no antibiotic therapy	Blood, 0 h (after suspicion of sepsis)	IL-6: flow cytometry (LUMINEX), PCT: immunol uminometric assay (LUMItest R PCT; BRAHMS Diagnostica, Berlin, Germany)	IL-6 + PCT	Either/or	IL-6: 250, PCT: 25 (specificity of the single marker ∼95%)	71	88	NA	65	91
							IL-6 + PCT	either/or	IL-6: 12, PCT: 5.7 (sensitivity and specificity of the single marker almost identical)	93	46	NA	35	95
Silveira and Procianoy, Brazil, (9)	EONS (≤5 days)	117 NICU preterm and term infants with suspected sepsis: 66 infected, 51 uninfected	(1) Positive blood and/or CSF culture and ≥3 categories of clinical sepsis or (2) negative cultures and ≥3 categories of clinical sepsis	PROM, but no complete criteria for clinical sepsis, no antibiotic treatment up to discharge from hospital, no hospital readmission (<1 month)	Peripheral blood, 0 h (after suspicion of sepsis), 82.9% at ≤24 h PNA	Quantitative sandwich enzyme immunoassay technique (Quantikine)	IL-6 + TNF-α	and/or	IL-6: 32, TNF-α: 1298.5 (ROC, NS)		NA	NA	60.7	90
Doellner et al., Norway, (2)	NS	113 NICU preterm and term infants: 24 infected, 89 uninfected	(1) Positive blood/CSF culture and clinical signs for sepsis/meningitis or (2) negative blood culture, ≥3 categories of clinical signs and abnormal laboratory results (CRP, I:T ratio) or (3) negative blood culture, respiratory symptoms, X-ray consistent with pneumonia and abnormal laboratory results	Initially suspected of having an infection (not confirmed)	Peripheral blood, on admission to the NICU or on the next day, >92% <4 days (PNA)	IL-6–dependent mouse hybridoma cell line B13.29 (clone B9), as described by Ng [(15) cite]	IL-6 + CRP	and/or	IL-6: 50 pg/ml, CRP: 10 mg/L (NA)	96	74	NA	49	99

NA, not available; NS, not specified; UV, umbilical vein; UA, umbilical artery; PNA, postnatal age; NICU, neonatal intensive care unit; CSF, cerebrospinal fluid; CRP, C-reactive protein; WBC, white blood count; PC, platelet count; ABC, absolute band count; EONS, early-onset neonatal sepsis; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value; GA, gestational age.

(37%). Another study combined IL-6 > 250 pg/ml and PCT > 25 ng/ml resulting in a sensitivity of 71% and a specificity of 88% at the time of sepsis suspicion (32). Steinberger et al. (17) using cord blood cutoff values of IL-6 > 15.85 pg/ml and PCT > 0.235 ng/ml reported sensitivity and specificity of 91.7 and 77.1%, respectively, with an excellent AUC of 0.915. Silveira et al. (9) found that IL-6 > 32 pg/ml and TNF-α > 12 pg/ml had a sensitivity of 98.5%. Unfortunately, they did not report the specificity of their biomarker combination. Some studies suggested the use of three inflammatory markers (17, 31, 38). Using single-parameter analysis, Steinberger et al. (17) suggested a combined use of cord blood PCT and IL-6 with serial determinations of CRP over the first days of life to rule out infection (17). Other combinations like hs-CRP, PCT, and IL-6 (38) or presepsin, PCT, and one proinflammatory cytokine, either IL-6 or IL-8 (31), were found to be superior to the individual markers. Using an early and sensitive marker like IL-6 for screening, and confirming sepsis suspicion with a late and specific marker like CRP, measured a few hours later, is effective in diagnosing EONS (7). The potential of such combinations, however, might rather lie in their simultaneous measurement at sepsis suspicion (32). Their counteractive dynamics suggest using an either/or combination (4). Findings suggest that it is possible to define high cutoff values, increasing the specificity of the single markers, because a satisfactory sensitivity can be reached over the biomarker combination (2, 32, 42).

Chiesa et al. (22) described the quality of IL-6 diagnostic accuracy studies as suboptimal, with missing information on key elements like design, conduct, analysis, and interpretation of test accuracy. Study designs like non-consecutive sampling of patients, retrospective data collection, and identification of patients by searching hospital records are prone to spectrum bias (22). Reporting the actual dates of when the study was performed allows the reader to consider any technological advances that have taken place in the meantime. If more than one reference standard is used to verify results of the index test, incorrectly treating their results as equivalent will lead to differential verification bias (48). Reported estimates of diagnostic accuracy are on average 60% higher than those found in studies that used a single reference standard (49). However, reference standards are not interchangeable as they may not have the same degree of error and may not identify the same segment of the disease (22). Consequently, the decision to not use positive blood culture as sole standard for diagnosing EONS in diagnostic accuracy studies has been described as arguable (4) and small numbers of culture proven sepsis cases within the study population are usually reported as limitation of the study (5, 19, 20, 32). The use of a mixed study population of culture-positive and clinical sepsis cases, however, is supported by the fact that the positive culture rate is extremely low in patients with EONS (19). This holds especially true for infants born to mothers who received antenatal or intrapartum antibiotics (43). Many studies comparing the two diagnostic subgroups found that IL-6 levels did not differ significantly (2, 10, 35, 46, 50, 51). FIRS might be associated with neonatal sepsis (28) but also with a neonatal systemic inflammatory response, which manifests as clinically suspected neonatal sepsis with negative blood and cerebrospinal fluid cultures (52). Adding a group of sick neonates without infection, in which IL-6 was also significantly increased, to healthy controls lessened the diagnostic value of IL-6 (14).

Some studies reported data of a group of patients in whom the applied diagnostic criteria resulted non-conclusive, so that sepsis could neither be excluded nor confirmed (2, 10, 33–35). IL-6 levels in these neonates were found to be higher than in uninfected sick controls, but lower than in neonates with infection (2). Termed uncertain sepsis (33, 35), infection unlikely (34), or mixed group (2), those groups were mostly excluded from study analysis and the determination of cutoff values. While not all studies provided this information (34), it was generally assumed in this analysis.

In diagnostic accuracy studies, it is of utmost importance to describe the populations from which patients and patient controls originated as well as the severity of sepsis within the patient group (22). Illness severity may alter the diagnostic value of IL-6 (35), and the use of illness severity scores has been included into the STARD checklist for assessing the quality of IL-6 accuracy studies (22). Chiesa et al. (35) used the Score for Neonatal Acute Physiology (SNAP) and its Perinatal Extension (SNAP-PE) (53). High IL-6 values in infants with EONS were independent of illness severity in contrast to uninfected infants in which higher IL-6 levels correlated with higher SNAP scores. Similar results were reported by Labenne et al. (7). Messer et al. (10) found no correlation between the magnitude of IL-6 levels and the severity of infection. These findings indicate that illness severity does not influence IL-6 levels in infected infants but leads to increased levels in uninfected infants, affecting the specificity of a diagnostic test relying on IL-6.

If the result of the index test influences the decision to order the reference test, measures of diagnostic accuracy will be biased

TABLE 4 | Subgroup analysis of IL-6 diagnostic accuracy studies on EONS.

Subgroup			No. Studies	Pooled sensitivity, %	Pooled specificity, %
Study population	Preterm	All	13	83	82
		<30 pg/ml	6	80	81
		≥30 pg/ml	7	84	82
	Preterm and term	All	18	73	82
		<80 pg/ml	9	73	71
		≥80 pg/ml	9	73	90
Sample and timing	Cord blood	All	18	83	85
		UV	5	87	83
	Peripheral blood	All	12	71	77
		<48 h	6	80	77
		<1 week	6	64	77
Biomarker combinations	IL-6 + CRP		3	84	61

UV, umbilical vein.

TABLE 5 | Quality of IL-6 accuracy studies for diagnosing early-onset neonatal sepsis from 1990 to 2020 according to the STARD criteria (Standards of Reporting Diagnostic Accuracy Studies).

Quality of reporting of IL-6 accuracy studies for diagnosing early-onset neonatal infection		
Category and item no.	YES	NO
Methods—participants		
Describe the study population:		
1A. The inclusion and exclusion criteria	22	9
1B. Setting, and locations where data were collected	31	0
Describe participant recruitment:		
2A. Was enrollment of patients based only on clinical signs suggesting infection?	9	22
2B. Were such patients consecutively enrolled?	2	7
2C. Was enrollment of patients based only on maternal risk factors for infection?	7	24
2D. Were such patients consecutively enrolled?	3	4
2E. Were patients identified by searching hospital records?	2	29
2F. Did the study include both patients already diagnosed with sepsis and participants in whom sepsis had been excluded?	3	28
Describe data collection:		
3. Was data collection planned before the index test and reference standard were performed (prospective study)?	15	16
Test methods		
Methods pertaining to the reference standard and the index test:		
4A. Was a composite reference standard used to identify all newborns with sepsis, and verify index test results in infected babies?	29	2
4B. Was a reference standard used to exclude sepsis?	14	17
4C. Was a composite reference standard used to identify all newborns without sepsis, and verify index test results in uninfected babies?	3	11
4D. Did the index test or its comparator form part of the reference standard?	10	21
5. Were categories of results of the index test (including cutoffs) and the reference standard defined after obtaining results?	29	2
6. Did the study report the number, training, and expertise of the persons executing and reading the index tests and the reference standard?	7	24
7. Was there blinding to results of the index test and the reference standard?	11	20
Statistical methods		
8. Describe the statistical methods used to quantify uncertainty (i.e., 95% confidence intervals)?	5	26
9. Describe methods for calculating test reproducibility	14	17
Results—participants and test results		
10A. Describe when the study was done, including beginning and ending dates of recruitment	28	3
10B. Did the study report clinical and demographic (postnatal hours or days, gestational age, birth weight, gender) features in those with and without sepsis?	22	9
10C. Did the study report distribution of illness severity scores in those with and without sepsis?	3	28
11. Report the number of participants satisfying the criteria for inclusion that did or did not undergo the index tests and/or or the reference standard; describe why participants failed to receive either test.	11	20
12. Report a cross-tabulation of the results (including indeterminate and missing results) by the results of the reference standard; for continuous results report the distribution of the test results by the results of the reference standard	23	8
Results—estimates		
13. Report measures of statistical uncertainty (i.e., 95% confidence intervals)	5	26
14. Report how indeterminate results, missing responses and outliers of index tests were handled	8	23
15. Report estimates of test reproducibility	14	17

(54). Incorporation bias occurs if the index test or the comparator of the index test form part of the reference standard (54, 55). This gives the person interpreting the results of the index test or its comparator some knowledge of the results of the reference standard (22). CRP was part of the reference standard for sepsis diagnosis in most of the included studies. Not only does this fact distort the diagnostic abilities of CRP when used as a comparator of the index test, but also biomarker combinations including CRP and markers related to CRP are biased (32). Therefore, blinding to both the index test and the knowledge of its outcome should be performed to avoid test review and diagnostic review bias (22).

In the included studies, cutoffs were mostly defined *post hoc* using ROC analyses and Youden's index. While Youden's index has the advantage of being a single measure, it loses the distinction between the sensitivity and specificity of a test. So do other error-based measures like the area under the ROC curve, an estimator of the overall accuracy of a test (17). Defining the cutoffs after the results are obtained reduces the likelihood that another study will replicate the findings (22).

Strengths of the Study

The type of sepsis has been identified as a significant source of heterogeneity (p = 0.0351) through a subgroup analysis conducted by Qiu et al. (25). This was apparent even though their subgroups were formed by a group of early-onset sepsis cases and

a mixed (i.e., early/late-onset) sepsis group. We eliminated this factor by including only cases of EONS in our meta-analysis.

Limitations of the Study

There are several important limitations to our systematic review and meta-analysis. First, we limited our database search to PubMed, which might have yielded a biased sample of primary studies and, thus, may influence the accuracy of summary effects. We did, however, check the reference lists of included studies and other important systematic reviews, to include other relevant studies. We did not include unpublished data and data reported in abstract form, which may result in publication bias. To investigate possible sources of heterogeneity within IL-6 diagnostic accuracy studies, we included a heterogeneous group of studies in this meta-analysis. While this gave us a sufficient number of studies ($\geq$5 studies in each subgroup, with the exception of biomarker combinations) for meaningful subgroup analysis of multiple possible influencing factors, it might have compromised the precision of our study, due to remaining sources of heterogeneity within the subgroups. Exploration of a specific source of heterogeneity within an otherwise homogenous subgroup might be subject for future research. The small number of studies looking at biomarker combinations limited our attempt to give more information on their potential, as it did not allow for subgroup analysis. Finally, initial data selection and collection were performed by reviewer JE only; the final decision however was based on a discussion with reviewer BR and resolved by consensus.

CONCLUSION

We identified 31 studies on IL-6 diagnostic accuracy for EONS diagnosis between 1990 and 2020 including 3,276 infants. The range of IL-6 sensitivities and specificities in neonatal samples was 42.1–100% and 43–100% with median values of 83 and 83.3%, respectively. IL-6 accuracy was better in preterm infants than in mixed term and preterm infants. The sensitivity and specificity in umbilical cord blood were higher than in neonatal peripheral blood, 83 vs. 71% and 85 vs. 77%, respectively. Diagnostic accuracy in peripheral blood was higher if blood was drawn within the first 48 h. The combination of IL-6 and CRP had a sensitivity in the range of cord blood IL-6 as single measure (84 vs. 83%), but far lower specificity (61 vs. 85%). Finally, quality assessment by the STARD criteria revealed poor quality of the majority of studies; thus, we need better designed, prospective, multicenter investigations on IL-6 and its use for the prediction of EONS.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

JE and BR contributed to conception and design of the study and wrote sections of the manuscript. JE performed the database search, study selection, data extraction, and statistical analysis and wrote the first draft of the manuscript. Both authors contributed to manuscript revision, read, and approved the submitted version.

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