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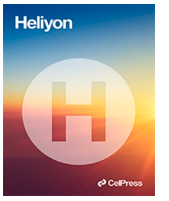

Review article

Neutrophil extracellular traps in adult diseases and neonatal bacterial infectious diseases: A review

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ABSTRACT

Neutrophils, the most abundant type of white blood cells, are pivotal in fighting bacterial infections due to their immunological and anti-infection capabilities. In recent years, scientists have discovered a novel mechanism known as neutrophil extracellular traps, which are fibrous networks primarily released by neutrophils that combat bacterial infections. There is a growing interest in studying NETs and their role in human infectious diseases, particularly in neonates susceptible to bacterial infections. NETs and their components have been found in various samples from neonatal-infected patients, providing a new route for early diagnosis of neonatal infectious diseases. This paper aims to summarize the studies on NETs in adult diseases and mainly discuss NETs in neonatal sepsis, necrotizing enterocolitis, and purulent meningitis, to provide scientific evidence for early monitoring, diagnosis, and treatment of neonatal infections.

1. Introduction

Globally, prematurity is the leading cause of infection in newborns due to relatively immature immune systems, long-term hospitalization, and increased susceptibility to fatal infections [1]. In 2010, there were over 15 million cases of preterm birth (gestational age <37 weeks), with an incidence rate of 11.1 % [2]. Although there was a decrease in the global preterm birth rate to 10.6 % in 2014 [3], it has been increasing in China in recent years [4]. Most deaths under 5 years of age between 2000 and 2019 occurred during the neonatal period, with preterm complications, perinatal events, and sepsis or meningitis being the leading causes. Preterm birth, infection, and asphyxia are the three primary causes of neonatal death worldwide [5–7]. Bacterial infections are a common cause of newborn infection. Clinical symptoms are frequently nonspecific, and there is currently no reliable biomarker for diagnosis. Early antibiotic administration is complicated, leading to severe consequences and high death and disability rates [8]. Neutrophils, the most abundant white blood cells during bacterial infections, possess immune and anti-infection properties [9,10]. While some biomarkers, such as white blood cell count (WBC), hypersensitive C-reactive protein (CRP), and procalcitonin (PCT), have been proposed [11,12], they lack specificity and accuracy. Neutrophil extracellular traps (NETs) have recently gained attention as a new antibacterial mechanism. NETs are fibrous networks composed of decondensed chromatin or circulating free DNA (cf-DNA). Histones and granular proteins, such as myeloperoxidase (MPO) and neutrophil elastase (NE), are also present in NETs. Research conducted in adults has demonstrated that during severe infections, there is a notable increase in the levels of NETs. These heightened levels have been found to possess significant diagnostic value, indicating their potential as a diagnostic marker for severe infections in adult patients. With

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increasing clinical and fundamental research on NETs in neonatal bacterial infections, this paper aims to provide a comprehensive summary and discussion of their application and the progress of clinical research in this field.

2. Mechanism of NETs formation

In bacterial infections, neutrophils are the first white blood cells to accumulate at the infected site. As part of the innate immune system, neutrophils eliminate pathogens through various mechanisms [10]. Phagocytosis, degranulation, or the production of substances such as reactive oxygen species (ROS)- were originally believed to be the primary mechanism. However, the discovery of NETs in 1996 [13] and their detailed description by Brinkman in 2004 proposed a novel anti-infective mechanism of neutrophils [14]. When the body is infected, NETs can effectively capture and restrict the spread of pathogens by forming a skeleton-like structure at the site of inflammation. Furthermore, NETs provide a concentrated source of antibacterial molecules that can directly eliminate pathogens [14,15]. To date, two mechanisms for the formation of NETs are well known. One mechanism by which neutrophils activate to produce NETs is called NETosis, a form of cell death distinct from apoptosis and necrosis [16,17]. NETosis is dependent on ROS generation by an enzyme called NADPH oxidase. Various stimuli such as *Lipopolysaccharide* (LPS), phorbol 12-myristate 13-acetate (PMA), cytokines, or immune complexes activate neutrophils via the protein kinase C (PKC)-Raf/MERK/ERK signaling pathway [14,18,19]. This pathway reduces nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, an enzyme responsible for generating ROS [20]. MPO is an enzyme found primarily in neutrophil granules. It utilizes hydrogen peroxide produced by the NADPH oxidase complex as a substrate. MPO catalyzes the oxidation of chloride ions (Cl^-) to hypochlorous acid (HOCl) and other reactive intermediates [21]. The presence of hydrogen peroxide, generated through NADPH oxidase-mediated superoxide production, promotes the release of NE from the granules [22]. During this process, NE degrades the actin cytoskeleton and translocates from the neutrophil granules into the

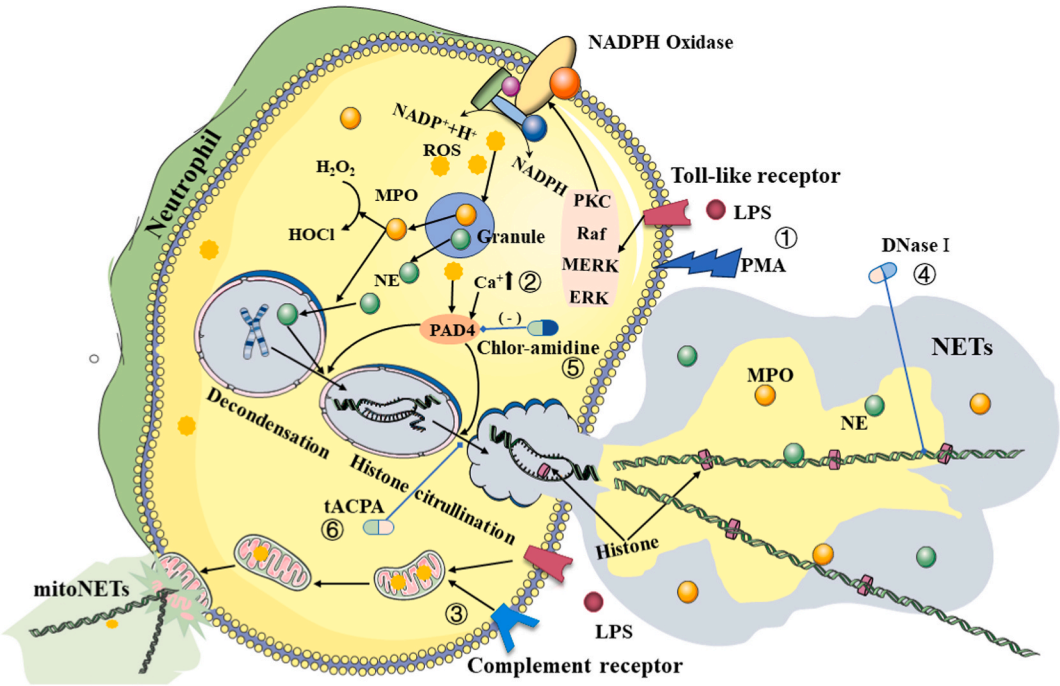

Fig. 1. NETs formation mechanism and Target therapeutics

1. Mechanism of NETs formation. ①Toll-like receptors recognize various stimuli such as LPS and PMA activating NADPH oxidase via the PKC-Raf/MERK/ERK signaling pathway and consequent ROS generation. ROS stimulates MPO and NE released from granules and migrated into the nucleus, causing chromatin decondensation and nuclear membrane disruption. Nuclear DNA, CitH3, and other protein granules are released into the extracellular space, releasing NETs. ②Elevated intracellular calcium levels or mitochondrial ROS can activate PAD4 which citrullinates histones and promotes chromatin decondensation by decreasing the electrostatic binding force between histones and DNA. ③mitoNETs: LPS recognition by toll-like receptors or complement receptors recognizes complement, producing mitochondrial ROS. Mitochondria translocate to the cell surface, degranulate protein particles, and release mitochondrial DNA into extracellular space. **2.NETs therapeutic targets.** ④DNase I: an enzyme that can hydrolyze the phosphodiester bonds of DNA molecules, leading to the dismantling and dissolution of the NETs structure, and reducing the activity of MPO and NE within NETs. ⑤Chlor-amidine, a PAD4 inhibitor may be a therapeutic target, reduced the neutrophil count, decreased levels of CitH3, and inhibited NET formation and tissue damage. ⑥tACPA: tACPA possesses the ability to selectively bind to citrulline residues on histones and hinder histone citrullination and subsequent chromatin decondensation, inhibiting the formation of NETs. LPS: lipopolysaccharide; PKC: protein kinase C; Raf: rapidly accelerated fibrosarcoma; MERK/ERK: mitogen-activated protein kinase; extracellular signal-regulated kinase; NADPH: niacinamide adenine dinucleotide phosphate; ROS: reactive oxygen species; MPO: myeloperoxidase; NE: neutrophil elastase; PAD4: protein arginine deiminase 4; CitH3: citrullinated histone H3; tACPA: therapeutic anti-citrullinated protein antibody.

nucleus, partially clipping histones to promote the decondensation of chromatin and the release of NETs [23,24]. Recent studies have shown that mitochondrial ROS production or after receptor stimulation of toll-like receptor 4, neutrophils can release mitochondrial DNA, along with other cellular contents, as part of the mitoNETs formation process [25]. While NADPH oxidase-dependent ROS production is a well-known trigger for NET formation, recent studies have revealed that other mechanisms involve the NADPH oxidase-independent pathway [26].The flow of extracellular calcium ions and the regulation of mitochondrial ROS through the small conductance potassium channel 3 (SK3) [27]. When there is an influx of calcium ions into the cytosol of cells, it activates peptidyl arginine deiminase 4 (PAD4), an enzyme involved in protein citrullination. PAD4 catalyzes the conversion of arginine residues in histone proteins, particularly histone 3 (H3), into citrulline. The citrullination of histone proteins has several effects, including weakening the positive charge associated with arginine residues. decreasing the electrostatic binding force between histones and DNA, leading to chromatin decondensation [28,29]. This process results in cell rupture and the release of nuclear DNA, citrullinated histone H3(CitH3), and other protein granules into the extracellular space, producing NETs (Fig. 1.).

The earlier processes collectively refer to NETosis, which is called lytic NETs [30]. NETosis generally takes around 3–4 h to complete [31]. Additionally, it has been discovered that the rapid formation and release of NETs can occur within 5–60 min without the need for neutrophil death [32]. Importantly, this process is independent of the ROS produced by the enzyme NADPH oxidase. Pathogenic microorganisms trigger the release of NETs through recognition by toll-like receptor 2 (TLR2), toll-like receptor 4 (TLR4), or complement receptors [33,34], along with PAD4 activation and NE translocation to the nucleus. The chromosomes, DNA, and nucleosomes within the neutrophil become scattered, resembling bead-like structures. Simultaneously, the nuclear membrane undergoes budding, forming nuclear vacuoles. Eventually, the neutrophils transform into enucleated cells. Additionally, within the cytoplasm, one can observe the presence of dense granules. Along with the nuclear vesicles, these granules are expelled to the cell's exterior through exocytosis. Once outside the cell, the contents intermix, leading to the formation of NETs [32]. These NETs are then secreted through vesicles in a process known as non-lytic NETs. Unlike lytic NETs, this process does not involve neutrophil lysis and

Table 1
Summary of NETs in animal study and human Diseases.

Disease	Year	Title	Study design	Objects	Conclusion	Reference
Sepsis	2008	Neutrophil-derived circulating free DNA (cf-DNA/NETs): a potential prognostic marker for posttraumatic development of inflammatory second hit and sepsis	A prospective pilot study that cf-DNA was directly quantified in plasma	37 multiple trauma patients	Cf -DNA/NETs seem to be a valuable additional marker for the calculation of injury severity and/or prediction of the inflammatory second hit	[38]
Sepsis	2017	Plasma level of neutrophil extracellular traps in septic patients and its clinical significance: a prospective observational study	A prospective observational study aimed to investigate the changes of NETs in sepsis patients and judge its value for early diagnosis.	23 sepsis patients, 20 non-sepsis patients	Cf-DNA/NETs increased significantly in sepsis patients than in WBC	[39]
endotoxic shock	2017	CitH3: a reliable blood biomarker for diagnosis and treatment of endotoxic shock	A basic study to further quantify CitH3 in the NETs in mouse models of both LPSS and HS.	Mice	CitH3 is a reliable biomarker than PCT and IL-6, due to its early appearance, specificity, duration, and response to therapeutic intervention.	[40]
SLE	2014	Elevated Plasma cf-DNA May be Associated with Active Lupus Nephritis and Partially Attributed to Abnormal Regulation of Neutrophil Extracellular Traps (NETs) in Patients with Systemic Lupus Erythematosus	A retrospective cohort study tested that elevated plasma cf-DNA levels are related to LN.	54 SLE patients and 43 control individuals	The cfDNA concentration was significantly higher between the SLE group and the healthy control group and associated with active LN	[41]
IBD	2018	Neutrophil extracellular traps in pediatric inflammatory bowel disease	A retrospective study aimed to describe the involvement of NETs in pediatric IBD and examined biopsies from the small bowel and colon.	12 patients (6 with CD,6 with UC) and 2 people with normal colonoscopy	NETs were found in biopsies taken from the small bowel and colon of pediatric patients with IBD and not in the samples from the two controls.	[42]
GC	2020	Diagnostic, Therapeutic Predictive, and Prognostic Value of Neutrophil Extracellular Traps in Patients with Gastric Adenocarcinoma	A clinical study determined whether NETs may serve as biomarkers in GC patients.	290 GC patients, 70 benign gastric disease patients, and 85 healthy controls	NETs have a novel diagnostic, therapeutic predictive, and prognostic value than CEA and CA19-9 in GC patients as a biomarker	[43]
Breast cancer	2020	Neutrophil Extracellular Traps Associate with Clinical Stages in Breast Cancer	A prospective study aimed to associate NETs with clinical stages of breast cancer.	45 female breast cancer patients	NETs increase in proportion to the stage of the disease, and higher levels of NE-DNA complexes in regional and metastatic disease	[44]

Annotation: LPSS: lipopolysaccharide (LPS)-induced shock; HS: hemorrhagic shock; IBD: inflammatory bowel disease; CD: Crohn's disease; UC: ulcerative colitis; SLE: systemic lupus erythematosus; LN: lupus nephritis; GC: gastric cancer.; CEA: carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9.

allows them to remain active in microorganism uptake [17,26,30]. However, the mechanism behind the formation of non-lytic NETs has not been extensively studied.

3. NETs in adult diseases

There is growing evidence that NETs play an essential role in various diseases, including infectious diseases, chronic aseptic inflammation, autoimmune diseases, thrombosis, and multiple types of tumors [30,35,36]. As previously mentioned, NETs can form rapidly, making it critical to standardize their detection, visualization, and establish a reliable reference range that can be a screening tool for clinical diagnosis and treatment [37] (Table 1.). Differentiating DNA and proteins associated with NETs from those derived from other cell death mechanisms can be challenging, given the potential for overlap in biochemical and cellular characteristics. However, a combination of morphological analysis, marker identification, and functional assays can assist in distinguishing NETs from other forms of cell death.

Interestingly, numerous basic studies have demonstrated the potential to detect NETs and NET-related components using microscopy, DNA staining technology, enzyme-linked immunosorbent assay (ELISA), and flow cytometry [45,46]. For instance, microscopy remains the most widely used approach for in vitro detection of NETs, involves immunostaining neutrophil-derived proteins (e.g., MPO and NE) in combination with DNA staining using a DNA-specific dye such as Sytox Green or DAPI [45]. The co-localization of neutrophil-derived proteins and cf-DNA indicates the presence of NETs. Identifying neutrophil-specific markers, such as NE, MPO, and PAD4, can provide evidence of NETosis. These markers can be detected using techniques such as ELISA and flow cytometry [37]. By employing these methods, researchers can effectively assess the presence and extent of NETosis, suggesting that early disease diagnosis is possible by differentiating them from other products derived from dead cells. Currently, various experimental methods to evaluate the formation of NETs have been demonstrated in animal and clinical trials. The most common approach involves the detection of cf-DNA, CitH3, MPO, or NE, which are the primary components of NETs. These components can be detected individually or in combination, showcasing the feasibility of assessing NETs formation using different techniques [37]. These novel markers offer promise for aiding in disease diagnosis, treatment, and prognosis.

Infectious diseases are one of the leading causes of rising hospitalization rates worldwide. Early infection management is essential for disease treatment [47,48]. It has been reported that NETs have been found to benefit from controlling bacterial, fungal, viral, and parasitic infections in adult infectious diseases [49]. In recent years, the level of NETs in peripheral blood has increased in adults with severe infection, which appears to have a high diagnostic value for infectious diseases [50,51]. Several investigations on adult patients with sepsis have shown a close relationship between the concentration of NETs in the blood and infection [52]. For instance, Margraf et al. [38] discovered high quantities of NET-related components in plasma, particularly cf-DNA, a biomarker of post-traumatic sepsis. In a recent prospective clinical study, it was also observed that the level of plasma neutrophil-derived cf-DNA (cf-DNA/NETs) was significantly higher in the sepsis group compared to the non-sepsis group and healthy control group (453 ± 185.37 μg/L vs. 188.35 ± 29.66 μg/L, 203.83 ± 43.25 μg/L, $p < 0.05$). This suggests that increased levels of cf-DNA/NETs may serve as a distinguishing factor between septic and non-septic patients. Furthermore, the diagnostic performance of plasma cf-DNA/NETs, as measured by the area under the receiver operating characteristic (ROC) curve (AUC), surpassed that of common clinical infection indicators such as WBC (AUC: $0.981 > 0.663$) [39]. These findings further support that increasing the diagnostic value of plasma NETs produced by neutrophils in sepsis is more significant than that of WBC and can be used as an early biomarker. However, cf-DNA may also be derived from other cells other than NETs under pathological conditions. Combined detection of MPO/cf-DNA polymer in blood, as well as CitH3 alone or in combination with cf-DNA, has a higher specificity for detecting NETs [53,54]. In a subsequent animal study, Pan et al. [40] demonstrated that CitH3 is released into the bloodstream from NETs during severe infections. CitH3 in the blood of septic shock mice was highly specific, whereas CitH3 was not detected in the blood of hemorrhagic shock mice. CitH3, PCT, and IL-6 levels in serum were measured using ELISA at 0.5, 3, 12, and 24h after LPS injection. The detection of circulating CitH3 was rapid, with detectable levels observed 0.5 h after exposure. The levels of CitH3 reached their maximum accumulation at 12 h and remained elevated for a duration of 24 h. Compared to PCT and interleukin-6 (IL-6), CitH3 is a more sensitive early biomarker for endotoxic shock. Elevated levels of CitH3 in the bloodstream can also serve as a biomarker for severe infections and aid in the diagnosis and monitoring of diseases.

In addition, NETs have been implicated in the pathogenesis of both autoinflammatory and autoimmune diseases, including inflammatory bowel disease (IBD), vasculitis, systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA) [54,55]. In these conditions, NETs and their components can stimulate the production of autoantibodies, which can subsequently bind to neutrophils and trigger NETosis [56,57]. According to new research, an imbalance in NETosis and NET degradation can contribute to the inflammation and tissue damage observed in autoimmune disease and add to the severity of this disease activity [58–61]. NETs have been discovered to play a significant role in the pathophysiology of chronic aseptic inflammation, such as IBD, in multiple investigations [62,63]. IBD, which includes ulcerative colitis (UC) and Crohn's disease (CD) [63], is associated with an inflammatory response, and the presence of NETs in patients with IBD has been extensively studied using various techniques, including metaproteomic analysis and histological examination. In a specific study, fecal samples from patients diagnosed with CD and UC were subjected to metaproteomic analysis, which revealed the existence of proteins that constitute NETs [64]. Additionally, Gottlieb et al. [42] conducted a study examining intestinal biopsies from pediatric IBD patients and confirmed the presence of NETs in samples obtained from both pediatric CD and UC patients. This research has shown that patients with IBD have increased levels of MPO and NE, both of which are components of NETs that can differentiate IBD from non-inflammatory diseases like irritable bowel syndrome. However, there is currently insufficient evidence to distinguish between UC and CD. Most of the research conducted on NETs and autoimmune diseases has focused on their association with adult populations, particularly in the setting of Anti-neutrophil cytoplasmic

Table 2
Summary of NETs in animal and neonatal bacterial infectious disease.

Diseases	Year	Title	Sample collection	Study design, Objects	NET-marker	Conclusion	Reference
NEC	2012	Fecal calprotectin levels are increased in infants with necrotizing enterocolitis.	Feces at diagnosis and 3 days later	A prospective controlled study on 25 preterm infants with stage 2–3 NEC and 25 controls	fecal calprotectin using ELISA	Fecal calprotectin increases in infants with NEC and serial measurements may be useful as a noninvasive prognostic marker for disease progression.	[79]
Neonatal sepsis and NEC	2017	Elevated levels of circulating cell-free DNA and neutrophil proteins are associated with neonatal sepsis and necrotizing enterocolitis in immature mice, pigs, and infants	Preterm pigs plasm and the small intestine, neonatal mice plasm and spleen tissue, and preterm infants blood	Animal experimental on 144 preterm pigs, 11 S<superscript></superscript> E<superscript></superscript> pigs and 10 SAL pigs, 12 S<superscript></superscript> E<superscript></superscript> mice and 9 SAL mice; Retrospective study on 27 neonates with LOS and/or NEC and 27controls	cfDNA using the Quant-iT PicoGreen dsDNA Assay Kit	Elevated plasma cfDNA and neutrophil protein levels are associated with LOS and NEC diagnosis, with consistent data across preterm infants, preterm pigs, and neonatal mice.	[80]
NEC	2018	NEC is likely a NETs dependent process and markers of NETosis are predictive of NEC in mice and humans.	Blood and Intestine tissue	Animal experimental on 76 neonatal mice	cfDNA and DNase in plasma using Sytox Green; NE, MPO, CitH3 in intestine tissue using immunohistochemical staining	Serum surrogate markers of NETosis (such as cfDNA) appear to predict NEC in neonatal mice.	[81]
NEC	2019	Serial fecal calprotectin in the prediction of necrotizing enterocolitis in preterm neonates	Feces within 48h after birth and thereafter 2 times a week, during 5 weeks postpartum or until NICU discharge	A prospective case-control study on 100 preterm infants with a high risk for NEC	fecal calprotectin measured by ELISA	High concentrations and wide interindividual variations in calprotectin in preterm infants during the first weeks of life.	[82]
Neonatal sepsis	2019	Markers of NETosis Do Not Predict Neonatal Early Onset Sepsis: A Pilot Study	Umbilical cord blood immediately after birth	In a prospective study on term and preterm infants, 17 neonatal EOS and 17 controls without infection	cfDNA measured with immunofluorescence labeled by Sytox Green, MPO and NE quantified by ELISA	NET markers in umbilical cord blood do not predict neonatal sepsis onset.	[83]
Neonatal sepsis	2022	The expression and clinical significance of neutrophil extracellular trapping nets in neonatal sepsis.	Peripheral blood	Prospective research on 39 term infants with sepsis and 35 control groups	CitH3-DNA using ELISA; cfDNA measured by immunofluorescence	NETs levels increase significantly in neonatal sepsis patients, especially CitH3-DNA with a strong specificity, and can be considered as a biomarker for early diagnosis of neonatal sepsis.	[84]
Neonatal sepsis	2022	cfDNA and DNases: New Biomarkers of Sepsis in Preterm Neonates-A Pilot Study	Peripheral blood	A retrospective case-control study on 31 preterm infants: 3 EOS and 10 controls, 6 LOS and 12 controls	cfDNA measured with fluorescence-based assay using Sytox Orange Nucleic Acid Stain; DNaes I, MPO, NE measured using ELISA	CfDNA and DNase appear to be potential biomarkers for diagnosing early and late-onset neonatal sepsis in preterm infants.	[85]

Annotation: EOS: early-onset neonatal sepsis; LOS: later-onset neonatal sepsis; SE pigs(mice): pigs(mice) were systemically injected with S. epidermidis; SAL pigs(mice): pigs(mice) were systemically injected with saline.

antibodies (ANCA) associated vasculitis [65], ANCA-associated vasculitis refers to a group of autoimmune inflammatory diseases that affect small blood vessels throughout the body. ANCA is a specific type of antibody that can be detected in the plasma of patients suffering from small vascular vasculitis [66]. Early research has revealed that proteins found within neutrophils, such as MPO and proteinase 3 (PR3), can cause autoimmune ANCA production, ultimately damaging small blood vessels [67]. The current studies have also demonstrated that NETs contain MPO and PR3, which are targeted by ANCA. There is an association between ANCA and the activation of NETs formation in patients [68–70]. A specific case-control study has revealed that patients with ANCA-associated vasculitis have a higher formation ability of NETs in their serum than healthy controls. Additionally, the formation of NETs is positively correlated with disease activity in ANCA-associated vasculitis. Moreover, the presence of NETs has been associated with an increased risk of venous thrombosis in these patients [69,71]. Similarly, in patients with SLE, specific proteins within NETs undergo modifications, leading to the production of autoantibodies against double-stranded DNA (dsDNA). These autoantibodies form immune complexes that are deposited in the skin and kidneys, resulting in tissue damage [56,60]. According to the study by Zhang et al. [41], an analysis of NETs in 54 SLE patients compared to 43 control individuals revealed that the mean blood concentration of cf-DNA was significantly higher in SLE patients (236.66 $\pm$ 40.09 ng/mL vs. 187.96 $\pm$ 40.55 ng/mL, $p < 0.0001$). This higher cf-DNA concentration in SLE patients suggests an association with NETs formation. In the same study, the authors corroborated a positive correlation between cf-DNA concentration and 24-h proteinuria, indicating kidney involvement and disease activity in SLE. These findings provide valuable insights into the role of NETs in autoimmune diseases and suggest the potential utility of NETs as biomarkers for diagnosing autoimmune diseases and assessing disease activity in the future.

Several studies have demonstrated the significant role of NETs in the development, progression, and metastasis of tumors [72,73]. In a clinical study of gastric cancer, the authors investigated the specific value of NETs in human gastric cancer biopsy and blood. They demonstrated that NETs have shown a better diagnostic value when compared with common serum biomarkers such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) [43]. In the subsequent study, combining the assessment of NETs with other biomarkers can lead to higher specificity and sensitivity in diagnosing malignant tumors [74]. The incorporation of NETs into diagnostic approaches may enhance the accuracy of tumor detection and improve early detection and treatment outcomes. In addition, Emerging research suggests that NETs play a role in the staging and progression of malignant tumors. Rivera-Franco et al. [44] reported that the level of NETs was associated with the clinical stage of breast cancer, with higher levels detected in metastatic sites than in primary lesions. Observational studies have also suggested that NETs play a crucial role in the progression and distant metastasis of colorectal cancer [74,75]. Therefore, NETs and their components serve as novel serum biomarkers for diagnosing various malignant tumors in the future. The involvement of NETs in tumor staging and progression highlights their potential significance as therapeutic targets and prognostic indicators in cancer. However, it needs to be noted that NETs may lack specificity for diagnosing a specific type of tumor.

4. NETs and neonatal bacterial infectious diseases

The progress in research on NETs in adult diseases has indeed provided valuable insights into neonatal disease detection, diagnosis, and prognosis. Despite belonging to the same cell type, adult neutrophils and neonatal neutrophils possess distinct characteristics that significantly influence neutrophil host defense and NETs formation [30]. Neonatal neutrophils, in comparison to their adult counterparts, display functional immaturity. This immaturity profoundly affects various aspects of neutrophil function, including chemotaxis and phagocytosis [76]. An important distinguishing feature is that neonatal neutrophils exhibit reduced levels of ROS production, primarily attributed to diminished expression and activity of crucial enzymes involved in this process. Consequently, these neutrophils have a limited capacity to generate ROS effectively, impeding their ability to eradicate pathogens. Furthermore, research has revealed that neonatal neutrophils display impaired NET formation compared to adult neutrophils [77,78]. This deficiency in producing NETs can compromise the ability of neonatal neutrophils to capture and eliminate pathogens, potentially contributing to the heightened susceptibility of newborns to infections. Neonatal infection is one of the most common health concerns, and studying the formation and mechanism of NETs during the early life stages is crucial for understanding the pathogenesis of these infections. Research in this area is ongoing and holds promise for advancing our understanding of neonatal bacterial infectious diseases (Table 2.).

4.1. Neonatal sepsis

Neonatal sepsis is a significant bacterial infection in newborns, especially preterm infants. It is a condition that can lead to mortality if not promptly diagnosed and treated. Neonatal sepsis is commonly categorized into two types based on onset time: early-onset neonatal sepsis (EOS) and late-onset neonatal sepsis (LOS). However, the clinical manifestations of both types are nonspecific, making it challenging to diagnose accurately. Therefore, identifying early biomarkers is essential to improve the prognosis and initiate timely treatment [86]. While previous research has demonstrated the reliability of neutrophil-related biomarkers like cf-DNA and protein in diagnosing sepsis in adults, there is growing interest in their utility for neonatal sepsis as well. In a study by Colón et al. [87], it was found that the expression of NETs in children with sepsis was higher compared to adults. Additionally, there was a positive correlation between the severity of sepsis and the concentration of NETs. These findings suggested that the concentration of NETs in children's serum can also serve as an indicator of sepsis, further emphasizing the significance of NETs in evaluating sepsis in pediatric patients. Current research suggests that detecting NET-related components in peripheral blood can aid in the early diagnosis of sepsis in preterm infants. Liu et al. [84] conducted a prospective study on 74 term neonates (35 in the control group and 39 in the sepsis group). They found that NETs were highly expressed in the peripheral blood of neonates with sepsis and could be used as an early biomarker. It is important to note that research in this area is still ongoing, and further investigations are needed to reconcile these

contradictory findings. A study utilizing preterm animal-induced sepsis models (mice and pigs) and LOS in preterm infants revealed consistent elevations in cf-DNA and neutrophil-associated protein levels across three different species. This finding suggests a potential association between cf-DNA and neutrophil-associated proteins in the diagnosis of LOS in preterm infants [80]. Another study by Lenz et al. [85] found significantly higher levels of cf-DNA, and CRP in the peripheral blood of preterm infants with EOS or LOS compared to the control group. These findings imply that cf-DNA from NETs have the potential to serve as diagnostic biomarkers for sepsis in preterm infants. While numerous studies have highlighted the significance of NETs in the early diagnosis of neonatal sepsis, other molecules present in different sample collections can affect the detection of NETs and related components. Stiel et al. [83] have demonstrated no significant association between NETs markers in cord blood and neonatal infection within 72 h postpartum, suggesting that NETs cannot predict neonatal EOS. One possible explanation is the inhibitory effect of the neonatal NET inhibitory factor (nNIF) in newborns' cord blood shortly after birth, which may prevent the formation or release of NETs in response to sepsis [88]. Additionally, there is currently no standardized diagnostic cut-off value for EOS and LOS in the literature, which suggests a potential area for future multi-center clinical research.

4.2. Necrotizing enterocolitis

Necrotizing enterocolitis (NEC) is a common gastrointestinal emergency that affects neonates, with an incidence ranging from 2 % to 5 % in neonatal intensive care units. Its morbidity and mortality are inversely proportional to birth weight and gestational age, with mortality rates as high as 30 %–50.9 % in very low birth weight infants [89–91]. Despite advances in treatment, the exact cause or pathogenesis of NEC is still not fully understood, contributing to the ongoing risk it poses to infants [92]. Timely intervention in infants weighing over 1500 g can lead to survival. Still, delayed diagnosis due to the lack of early diagnostic biomarkers for preterm infants with atypical clinical symptoms may result in advanced NEC that requires surgical treatment. Despite improvements in treatment guidelines and expert consensus, the morbidity and mortality associated with NEC remain high. Fecal calprotectin, a protein found in neutrophils and derived from NETs [93], has been recommended by the American Gastroenterological Association as an important diagnostic tool for IBD due to its high predictive value [94]. In the case of NEC, which involves inflammation and significant damage to the intestinal mucosal barrier, previous studies have shown that fecal calprotectin levels are higher in neonates with NEC compared to healthy neonates [93,79]. Fecal calprotectin has shown promise as a potential biomarker for diagnosing and monitoring NEC. However, it is crucial to consider that neonates typically have higher fecal calprotectin levels in their first week than older children and adults [82]. They were discriminating whether calprotectin found in NEC derived from NETs or other forms of neutrophil cell death poses a notable challenge. Neutrophils can release calprotectin through different mechanisms, such as necrosis, another condition of neutrophil cell death [95]. Consequently, interpreting calprotectin of calprotectin as a diagnostic tool for NEC becomes unclear. To gain more insight into the predominant source of calprotectin, immunofluorescence staining for specific NETs markers or microscopy can provide additional information regarding the presence and structure of NETs, enabling a more accurate determination of the predominant source of calprotectin. Recent research indicates that neonates with suspected NEC have significant NETs in their blood or intestinal tissue. Animal models of LOS and/or NEC have also demonstrated higher levels of NET-related components, indicating their potential involvement in the pathogenesis of these conditions. Retrospective studies on preterm NEC populations have consistently found a correlation between elevated cf-DNA levels in neonatal plasma and NEC diagnosis [80]. In another study by Vincent et al. [81], the presence of NET-related components was observed in blood and intestinal tissue samples of NEC mice and NEC children, respectively. The study showed that neutrophil activation and NET-related components were significantly elevated in NEC animal models and neonatal NEC samples compared to the control group, suggesting that NETs play a vital role in the development of NEC. cf-DNA, a primary NET-related component in serum, may be a reliable biomarker for NEC diagnosis. While there is evidence suggesting the involvement of NETs in the pathogenesis of NEC, further research is required to determine whether NETs can be detected in the feces of newborns with NEC and used as an early screening biomarker. Additionally, it is worth noting that NETs may not be able to differentiate NEC from other gastrointestinal infectious diseases, and their specificity in NEC diagnosis still needs to be confirmed through future studies.

4.3. Purulent meningitis

Purulent meningitis is the most prevalent bacterial infectious disease of the central nervous system in neonates. Due to immature humoral and cellular immunity and the imperfect development of the blood-brain barrier, premature infants are at a higher risk. In Western countries, the incidence of purulent meningitis ranges from 0.22 % to 0.25 %, with premature infants accounting for approximately 3 % of cases [96,97]. Like other neonatal infectious diseases, diagnosis is challenging due to a lack of specific clinical features, leading to high mortality rates and potential long-term neurological sequelae that impact the children's health. While positive cerebrospinal fluid (CSF) culture remains the gold standard for diagnosis, its limitations include low positive rates and time-consuming processes, resulting in delayed administration of sensitive antibiotics and potentially increasing mortality rates [96]. These challenges underscore the importance of further research to better understand the role of NETs in pathogenesis and to develop more effective diagnostic methods. A preliminary study on *Streptococcus suis* meningitis demonstrated the presence of NETs in the CSF of animal models [98]. However, there was no report of NETs in patients with acute bacterial meningitis in the CSF. Although recent studies have detected elevated levels of NETs in animal models with purulent meningitis, there are still uncertainties regarding the early diagnosis of patients. In recent years, studies have identified the presence of NETs in the CSF of patients with purulent meningitis infected by *Streptococcus pneumoniae* (pneumococci). Buhr and colleagues used immunofluorescence microscopy and mass spectrometry to detect NETs and NET-related proteins [98]. Similarly, Mohanty et al. [99] demonstrated the presence of NETs in the CSF of rats with S. suis

Table 3
Potential anti-NETs therapeutic target.

Target	Anti-NET therapeutics	Pharmacological molecules	Mechanism of action	Molecular weight	Reference
DNA matrixes	NET-derived DNA	Recombinant human deoxyribonuclease I (rhDNase I)	Degradation of NETs	37000Da	[101, 102]
PAD4	PAD4 inhibitor	chlor-amidine	Inhibition of PAD4 and histone citrullination required for NET formation	310.78Da	[103]
CitH3	histone citrullination inhibitor	therapeutic anti-citrullinated protein antibody (tACPA)	Bind to citrulline residues on histones and Inhibition of NET formation	NA	[104]
MPO	MPO inhibitor	PF-1355	Inhibition of MPO required for NET formation	NA	[105]
NE	NE inhibitor	Sivelestat	Inhibition of NE required for NET formation	434Da	[106]
ROS	ROS scavengers	N-acetylcysteine (NAC)	ROS and NETs inhibition	163.19Da	[107]

meningitis and pneumococcal meningitis and in the CSF of patients with pneumococcal meningitis. However, NETs were not detected in the CSF of patients with meningitis with viral meningitis or subarachnoid hemorrhage caused by other pathogens. As such, seeing NETs in CSF may help diagnose purulent meningitis caused by pneumococcal infection. It is worth noting that current studies are predominantly focused on patients with pneumococcal meningitis, and there is a limited number of studies in this area. It remains unclear whether NETs are present in the CSF of patients with other bacterial meningitis. Additional research is necessary to explore whether NETs can serve as a biomarker for diagnosing purulent meningitis caused by various bacterial pathogens. Despite multiple pieces of evidence that indicate the pivotal role of NETs in adult purulent meningitis, there is a limited number of studies on the presence of NETs in children. In 2019, Appelgren et al. [100] detected active NETs in the CSF of older children with central nervous system infections and found that elevated levels of NETs were associated with viral infections. This suggests that NETs may play a role in the immune response to various pathogens, including viral infections, in pediatric central nervous system infections. Hence, we hypothesize that NETs could be present in the CSF of neonates suffering from purulent meningitis. To comprehensively comprehend the intricate association between NETs and neonatal purulent meningitis, as well as to confirm the practicability of NETs as a diagnostic tool for this condition, large-scale multicenter clinical investigations are required in the future.

5. NETs targeted therapy

Although NETs play a crucial role in combating bacterial infections and have the potential as diagnostic biomarkers for various diseases, an excessive or dysregulated formation can cause harm to the body. In conditions such as sepsis, NETs can contribute to releasing various inflammatory mediators that exaggerate inflammatory responses and promote thrombosis. The protein components of NETs can also act as antigens, leading to autoimmune and autoinflammatory diseases. Therefore, targeting NETs and inhibiting their release or disrupting established structures may have therapeutic potential for conditions [54]. Moreover, NETs have shown promise as a target for therapy in malignant tumors [54,74] (Table 3.).

Deoxyribonuclease (DNase) is an enzyme that can hydrolyze the phosphodiester bonds of DNA molecules, breaking them down into smaller components and leading to the dismantling and dissolution of the NETs structure. The hydrolysis of DNA by extracellular DNase comprises two families: DNase I and DNase II, which possess slightly distinct biochemical properties but partially overlapping functions [108]. Recombinant human deoxyribonuclease I (rhDNase I) is a genetically engineered form of DNase I naturally produced by the human body and has shown promising potential in the clinical treatment of diseases [101]. Since 1993, rhDNase I has been used in clinics for cystic fibrosis [102], and animal trials have repeatedly demonstrated its effectiveness in treating breast, lung, and other malignant tumors [73].In addition to DNase I, targeting the key enzyme PAD, which is involved in NETosis, may also be a therapeutic approach. By reducing histone citrullination, PAD4 inhibitors can prevent the release of NETs [109]. Early animal studies have shown that inhibiting PAD4 may be a therapeutic target [110]. Some PAD4 inhibitors, such as chlor-amidine, have shown positive effects in reducing albuminuria and immune complex deposition in the kidneys of mice with lupus nephritis [103]. Chirivi et al. [104] demonstrated the broad therapeutic potential of therapeutic anti-citrullinated protein antibody (tACPA) in the animal study. Their study revealed that citrullination of histones, particularly at position 3 (Cit3) of histone 2A (citH2A) and histone 4 (citH4), plays a crucial role in the formation of NETs. Notably, tACPA can selectively bind to citrulline residues on histones. By doing so, these antibodies effectively hinder histone citrullination and subsequent chromatin decondensation, thereby inhibiting the formation of NETs. This mechanism holds significant promise in preventing the excessive release of NETs and the consequential tissue damage frequently observed in diverse autoimmune and inflammatory conditions [111]. Anti-NETs therapy and related pathways are quickly emerging as a therapeutic approach for various diseases [54], but some studies on inhibiting NETs release, such as MPO or NE inhibitors, ROS clearance, or partial neutrophils clearance, remain controversial [105–107,112]. Further research is needed to validate these results and evaluate their efficacy in human subjects. Unfortunately, there is no specific report or study on anti-NET therapy use in neonatal bacterial infectious diseases.

6. Conclusion and perspective

NETs have gained significant attention in diagnosing, treating, and prognosis clinical disorders as a critical component of the innate immune system. However, excessive production of NETs can have harmful effects, including exacerbating acute inflammatory reactions, promoting chronic inflammatory and autoimmune illnesses, and increasing the risk or progression of cancer [113]. Anti-NETs therapy has emerged as a potential therapeutic target for diseases, but further research is needed to understand its impact on the immune response. NETs have been found to play a crucial role in the immune response to neonatal bacterial infections. They serve as an effective strategy for early diagnosis of such diseases. While the understanding of NETs in the pathogenesis and diagnosis of neonatal sepsis is still limited, their use as a diagnostic biomarker for neonatal sepsis is a topic of ongoing debate. Limited investigations have shown that full-term neonates or premature newborns cannot release NETs. The production time is delayed compared to adults, largely due to the immature regulatory mechanism of NETs synthesis in neonates [114]. Early detection, diagnosis, and intervention of neonatal bacterial infectious diseases remain a scientific challenge. Conducting numerous basic and multi-center clinical studies is crucial to address this challenge. These studies should analyze different subtypes of neonatal diseases, such as early-onset/late-onset, different ages and gestational ages, and conduct comprehensive and standardized clinical quantitative evaluations of NETs formation mechanism, activation time, and antibacterial ability. Determining the standard reference value range of related biomarkers or detecting them in combination with other diagnostic tools can significantly contribute to the early diagnosis, assessment of disease progression, identification of new therapeutic targets, and improvement of prognosis in neonatal bacterial infectious diseases.

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Ethics statement

Review or approval by an ethics committee was not needed for this study because no data on patients or experimental animals was produced in the review article. Informed consent was not required for this study because no clinical data was produced in the review article.

CRediT authorship contribution statement

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Declaration of competing interest

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