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DOCTORAL RESEARCH

CD64 and CD169 as Translational Biomarkers for Discriminating Bacterial from Viral Infections in Febrile Infants ≤90 Days of Age:

From Immune Pathophysiology to Bedside Application

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1. INTRODUCTION

1.1 Background and historical perspective

Fever in the first months of life can be the only sign of a life-threatening invasive bacterial infection (IBIs)[1-3]. Thus, fever in infants younger than 90 days represents one of the most challenging clinical scenarios in pediatric emergency medicine[1-3].

This population is characterized by a unique combination of biological vulnerability and clinical uncertainty[4-8]. Young infants are at increased risk of serious bacterial infections (SBIs), including urinary tract infections (UTIs) and IBIs, specifically bacteremia and bacterial meningitis (BM). These conditions may progress rapidly and lead to significant morbidity and mortality if not promptly recognized and treated[1,3,8-10].

Over the past decades, the management of febrile infants has evolved from a highly conservative approach, driven by the risk of severe infections such as *Group B Streptococcus agalactiae* (GBS) and characterized by universal hospitalization and treatment, toward more selective strategies aimed at reducing iatrogenic harm associated with unnecessary care[11-13]. The epidemiology of bacterial infections has significantly changed over time, with *Escherichia Coli* now representing the leading cause of bacteremia and GBS remaining the predominant cause of BM, reflecting the impact of preventive strategies such as GBS screening and pneumococcal vaccination[11-12].

This vulnerability is largely explained by the immaturity of the neonatal immune system[4-8,14]. Innate and adaptive immunity are underdeveloped at birth, with impaired neutrophil function, reduced cytokine production, and altered antigen presentation, resulting in diminished pathogen clearance and limited ability to mount effective inflammatory responses.[4,7-8,14-15]. Consequently, clinical manifestations of infection in early life are often subtle and nonspecific, and fever may be the only presenting sign of a potentially life-threatening condition[6-7,16].

Early studies showed that clinical judgment alone was insufficient to reliably distinguish infected from non-infected infants, even those appearing well. These findings led to full sepsis work-up, empirical antibiotic therapy, and hospitalization, especially in neonates.[11-13,17-18]. Thus for decades, it has been accepted that newborns with fever without source (FWS), assumed at high risk of bacteremia and BM, should undergo empirical and invasive evaluations including laboratory exams, lumbar puncture (LP), broad-spectrum antibiotics, and hospitalization, pending exclusion of bacterial infection via culture results.[11-12,17-20].

This approach, rooted in maximizing sensitivity to IBI detection, has been a cornerstone of clinical practice for decades and continues to influence modern guidelines[1,12-13]. However, it has become

clear that this strategy, although safe, results in substantial overtreatment. The widespread use of invasive procedures, antibiotic exposure, and hospital admission in infants who do not have bacterial infection raises concerns regarding healthcare costs, antimicrobial resistance, and patient burden[3,19,21-23]. These considerations have driven efforts to refine risk stratification and optimize the balance between safety and resource utilization.

1.2 The paradox of the febrile infant

Despite the historically justified aggressive approach, epidemiological data consistently show a paradox in the management of febrile infants. Although these patients are treated as high-risk for bacterial infection until proven otherwise, most febrile episodes are caused by viral pathogens.[3,9,12,24-29].

Many studies on SBIs epidemiology have been conducted before routine immunization against *Haemophilus Influenzae* type b (Hib) and *Streptococcus pneumoniae* and before prenatal screening changes for GBS. Large prospective, multicenter studies report SBI prevalence between 9% and 18%, depending on population characteristics and definitions[1,9,16,18,25,30]. Within this range, IBIs account for a smaller but significant proportion, typically 2% to 5%, especially in patients ≤ 28 days old[1,16,18,31-35]. Compared with earlier studies, UTIs now occur significantly more than bacteremia and BM, accounting for 92-95% of SBIs[21,25,36]. Thus, these data emphasize the importance of routine urinalysis in febrile infants[29].

Viral infections account for most of the febrile presentations. Studies with systematic viral testing show high viral pathogen prevalence, with respiratory viruses, enteroviruses, and parechoviruses notably relevant in early infancy[3,27-29,37-42]. However, the clinical implications of a positive viral test remain uncertain, especially in infants within the first month of life, where its role in guiding further evaluation and management is debated[12].

Importantly, viral infection does not exclude the possibility of concomitant SBI/IBI, as co-infections are reported in a non-negligible proportion of cases.[24,27-28,41,43].

This epidemiological imbalance creates tension in clinical decision-making. On one hand, the potentially severe consequences of missed bacterial infections justify a cautious approach. On the other side, the high prevalence of viral causes means many infants undergo unnecessary invasive testing and treatment.[1-3,20].

This paradox is further amplified by clinical heterogeneity in viral infections. While many viral illnesses are benign and self-limiting, others may present with severe systemic involvement,

including central nervous system (CNS) manifestations, closely mimicking bacterial infection and complicating clinical assessment.[9,29,44,41]. Thus, clinicians face the challenge of distinguishing bacterial from viral infections and identifying viral infections that require closer monitoring. This is evident for enterovirus (EV) and human parechovirus (hPeV) infections, leading viral causes of CNS infection in neonates and young infants, causing sepsis-like illness, encephalitis, meningoencephalitis, myocarditis, or hyperinflammatory syndromes.[37-40,42,45-47]. Conversely, other viruses like influenza and Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) generally have a milder course, though rarely they can present with sepsis-like syndrome or abnormal inflammatory and hematologic profiles, complicating initial evaluation[3,41,48-50].

This uncertainty leads to a systematic tendency toward overtreatment. High rates of LP, antibiotic administration, and hospitalization have been reported, even among well-appearing infants[1,51]. Moreover, substantial variability in clinical practice has been documented across institutions, particularly in infants aged 29–60 days and 61-90, reflecting the absence of a universally accepted, fully reliable diagnostic strategy.[11-12,23,52].

1.3 *State-of-art* and Limitations of current approaches

1.3.1 Clinical algorithms and guidelines

Significant variation in the management of febrile infants exists both nationally and worldwide [1,12,17,23,52-54]. Risk stratification is a cornerstone of clinical decision-making, identifying low-risk infants who can be managed without LP or empirical antibiotic therapy[52-53]. Attempts have been made to develop clinical prediction rules, guidelines, scores, and algorithms to identify low-risk patients for SBI, reducing reliance on subjective clinical judgment[52-53]. Historical approaches, such as the Rochester, Philadelphia, and Boston criteria, aimed to maximize diagnostic safety but lacked modern biomarkers, resulting in high sensitivity but low specificity, leading to overtesting and routine LP use.[55-57]. Thus, more recent tools were developed, including National Institute for Health and Care Excellence (NICE) guidelines, Step-by-Step (SBS) approach, updated Pediatric Emergency Care Applied Research Network (PECARN) rule, and American Academy of Pediatrics (AAP) clinical practice guidelines[1-2,12,17-18,20,23,52,54]. These tools integrate clinical appearance, age, urinalysis, and laboratory biomarkers to identify infants with a low risk of SBI. While they have significantly improved risk stratification with high sensibility, their performance remains imperfect lacking in specificity, explecially in infants ≤ 28 days, still often leading to overtreatment[1-2,10,12,17,23,52-54,58].

As UTI is the most common SBI in this age group, urinalysis yield is highest compared to blood markers or other tests.[2,25,36,59-61]. Blood biomarkers better predict bacteremia or BM. Among these, procalcitonin (PCT) best discriminates low-risk infants for IBIs, but its accuracy is lower for SBIs.[62-63]. However, the value of these tests is incomplete and controversial, as the pathological thresholds for biomarkers vary across the proposed guidelines[52]. Commonly, the PCT cutoff of 0.5 ng/mL is empirically used, whereas the PECARN rule is among the few approaches to formally derive and validate an alternative threshold (<1.71 ng/mL) within a prediction mode.[23,63].

Moreover, there is no consensus on age thresholds in febrile infants, both in defining the upper age limit at risk, typically set at ≤60 or ≤90 days, and in identifying the subgroup needing an empirical full sepsis work-up, with some guidelines adopting a ≤28-day cutoff and others a more restrictive ≤21-day threshold[12,20,23,54]. Notably, these thresholds were established a priori based on age-related IBI incidence and have served for decades as a clinical decision-making standard[19]. Other age-stratifications have been proposed, but data paucity does not currently allow other cut-offs[2,11-12,18-19,20,23,30,52].

Febrile neonates have twice the rate of bacteremia and BM as febrile infants in their second month of life[19]. Meanwhile, the SBI rate in the 61-90-day age group is similar to the 29-60-day group, prompting recommendations like SBS or NICE to consider patients up to 90 days at high risk, unlike the AAP and PECARN, which focus on those ≤60 days[2,11-12,16-20,23,52,54]. This uncertainty has led some Authors to question whether all current management strategies should extend to older infants, given the similar risk of UTIs and significant risk of IBIs in infants aged 61–90 days[64-65]. However, it remains unclear whether these patients should undergo the same diagnostic testing, treatment, or hospitalization without specific evidence-based recommendations from national guidelines[64-65].

The PECARN rule showed high sensitivity for identifying low IBI risk infants and neonates[2,23]. However, external validation studies have revealed reduced performance, with missed IBI cases even among low-risk infants and overall lower diagnostic accuracy than derivation cohorts[51]. Similarly, the updated PECARN rule had high sensitivity but lower specificity for identifying febrile infants ≤28 days old with IBIs, with no missed BM cases[2,23].

Comparably, the SBS approach, although highly sensitive, does not completely eliminate the risk of missed IBI, especially in neonates ≤21 days old, and may vary depending on population and clinical context[1,20,52,66]. Moreover, performance may decline in early fever stages when standard inflammatory markers (IM) are evolving and may be falsely reassuring.[20,26,31,51,67-70]. Following a sequential approach similar to SBS, the Lab-score combines biomarkers and urinalysis. Initially

developed for children up to 3 years, it was refined and validated in younger infants, leading to a more tailored approach in those <3 months with optimized PCT and C-Reactive Protein (CRP) thresholds.

However, its sensitivity and specificity remain lower than those of other approaches[71-72].

In addition, studies evaluating the real-world implementation of clinical practice guidelines have shown that none achieves an optimal balance between sensitivity and specificity[52]. Highly sensitive approaches often result in extremely low specificity, leading to high rates of unnecessary interventions[30]. This limitation is intrinsic to all current algorithms, which prioritize avoiding missed bacterial infections over reducing overtreatment.

Notably, less invasive approaches have lower diagnostic performance. Tools like the updated Rochester Criteria and Yale Observation Scale often misclassify, especially in infants under 28 days, frequently categorizing those with bacteremia or BM as low risk.[17,32-33,73].

Thus, none of the current clinical practice guidelines showed ideal performance over others[30,52]. Moreover, guidelines for febrile infants do not apply to preterm infants or those with comorbidities, as they exclude these groups or classify them as high-risk a priori.[12,20,23,35,55-57,71-72,74].

Despite advances in risk stratification, the clinical approach remains conservative. Febrile infants, especially in the first weeks, are often managed as potentially septic until proven otherwise, as missing IBIs can be life threatening. This risk aversion leads to over-investigation and overtreatment to minimize missed infections.. Table 1 summarizes all the scores, algorithms, and methods discussed.

	Rochester Criteria[55]	Philadelphia Criteria[57]	Boston Criteria[56]	YOS[73]	SBS[20]	PECARN[23]	AAP[12]	NICE[54]	Lab Score[71-72]
	Low-risk stratification tools			*Clinical score*	*PCT-driven Algorithm*	*PCT-driven Prediction Rule*	*Guidelines*	*Guidelines*	*Biomarker Score*
Age Range	0-60d	29-60d	28-89d	0-24m	≤ 90d	≤60	8-60d	<5y	7d-36m
A priori **High Risk Age**	\	≤ 28d	< 28d	< 60d	≤ 21d	≤ 28d	< 8d *8-21d* *22-28d*	< 3m	< 3m
Central role of **Clinical appearance***	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
Suspected **viral infection**	Included	Included	Included	\	Excluded	Included	*Only RSV excluded*	Included	\
Comorbidities**	Excluded	Excluded	Excluded	\	Excluded	Excluded	Excluded	Included	Excluded
Prematurity < 37w GA**	Excluded	Excluded	Excluded	Not mentioned	Excluded	Excluded	Excluded	Included	Not mentioned
Biomarkers	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
Normal WBC *$x10^3/L$*	5,000-15,000	< 15,000	< 20,000		Not included	Not included	Not included	5,000-15,000	Not included
Normal CRP *mg/l*	\	\	\		< 20	\	< 20	<20	≤ 75 *if PCT ≤ 0.5* ≤ 24 *if PCT 0.5-2*
Normal PCT *ng/ml*	\	\	\		<0.5	≤1.71	<0.5	\	≤ 0.5
Normal ANC *$x10^3/L$*	< 1,500	\	\		< 10,000	≤4,090	4,000-5,200	\	
Routine **Urinalysis**	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes
Routine **Blood Colture**	No	No	No	No	Yes < 21d	No	Yes < 21d	Yes	No
Routine **Lumbar Puncture**	No	Selective	Mandatory in all	Not defined	Mandatory ≤ 21d	Avoidable in low-risk	Mandatory ≤ 21d	***	Not specified
Sensitivity (%)	92-94	98-100	98-100	70-80	92-99	96-98	95-98	90-95	85-95
Specificity (%)	40-55	30-45	30-45	50-70	40-50	55-65	50-70	60-75	70-80
Negative Predictive Value (%)	98-99	≈99	≈99	90-95	99-100	99-100	≈99	97-99	97-99
Missed IBI/SBI (%)	1.5-2.5 (SBI)	0.5-1 (SBI)	0.5-1 (SBI)	10-20 (SBI)	0.7-0.9 (IBI)	0.5-1 (IBI)	0.5-1 (IBI)	1.5-2.5 (SBI)	2-4 (SBI)

Table 1. Comparison of clinical risk-stratification algorithms and guidelines for the management of febrile infants. The table summarizes the main structural and performance characteristics of the nine approaches currently used or historically relevant in the evaluation of febrile infants. For each approach, the following features were reported: age range of applicability; a priori high-risk age thresholds requiring empirical full sepsis workup regardless of clinical appearance or biomarker values; role of clinical appearance in risk stratification; inclusion or exclusion of suspected viral infection; exclusion criteria for comorbidities and prematurity; use of laboratory biomarkers and their normal thresholds (WBC, CRP, PCT, ANC); indication for routine urinalysis, blood culture, and lumbar puncture; and approximate diagnostic performance (sensitivity, specificity, and negative predictive value) for detection of SBI or IBI, as applicable. The reported sensitivity, specificity, and negative predictive value ranges represent estimates derived from selected external validation and cohort studies and should not be interpreted as fixed performance values. Diagnostic performance may vary substantially depending on the study population characteristics, prevalence of SBI, age distribution, outcome definitions, and local epidemiological context. All performance estimates are approximate and intended for comparison purposes only. *Clinical appearance includes assessment of well- versus ill-appearing status using standardized or non-standardized observational criteria, depending on the algorithm. **Comorbidities and prematurity are defined and operationalised variably across approaches; most algorithms systematically exclude these populations or classify them a priori as high-risk. ***NICE recommends lumbar puncture in all infants <1 month, in all infants aged 1–3 months who appear unwell, and in those with abnormal WBC, without a specific age threshold for mandatory deferral. *AAP: American Academy of Pediatrics; ANC: absolute neutrophil count; CRP: C-reactive protein; GA: gestational age; IBI: invasive bacterial infection (bacteremia and bacterial meningitis); NICE: National Institute for Health and Care Excellence; NPV: negative predictive value; PECARN: Pediatric Emergency Care Applied Research Network; PCT: procalcitonin; RSV: respiratory syncytial virus; SBI: serious bacterial infection (including UTI, bacteremia, and bacterial meningitis); SBS: Step-by-Step; UTI: urinary tract infection; YOS: Yale observation score; WBC: white blood cell count.*

1.3.2 The historical role of lumbar puncture

LP remains a key diagnostic tool in febrile young infants when the risk of BM is not negligible, as subtle clinical presentations require cerebrospinal fluid (CSF) analysis to confirm or exclude infection and guide therapy[12,20,54,75-76]. However, evidence argues against the routine use of LP in all febrile infants[76]. In selected low-risk populations, particularly > 28 days of life, BM incidence is very low, while LP has drawbacks, including high rates of traumatic or unsuccessful procedures, CSF contamination risk leading to false positives, unnecessary hospitalizations, prolonged antibiotic exposure, increased healthcare costs, and parental distress[34,20,76-78]. A more conservative approach may reduce hospitalizations and length of stay for infants >28 days old without missing any IBIs[76-77]. Current guidelines differ in their recommendations, balancing diagnostic safety and procedural burden. Historically, conservative protocols included routine CSF analysis, while modern risk-stratification allows deferring LP in well-appearing, low-risk infants, particularly >21 or >28 days old, depending on the approach[12,20,23]. In contrast, the NICE guidelines adopt a precautionary stance, recommending LP in all febrile infants under 1 month, all 1–3-month-olds who appear unwell, and those with abnormal white blood cell (WBC) counts[54].

The debate is particularly relevant in infants with documented UTIs, in whom concomitant meningitis appears rare but not negligible. Available evidence suggests that LP may be safely

deferred in carefully selected, well-appearing cases, although this remains controversial especially in neonates and in the presence of clinical or laboratory risk factors[79-80].

1.3.3 Static biomarkers in a dynamic disease

IMs are central to most clinical algorithms. Commonly used parameters include WBC count, absolute neutrophil count (ANC), CRP, and more recently PCT[12,20,23,63]. Urine, blood, and CSF cultures are also considered, with positivity occurring in approximately 24-48 hours[81].

However, their diagnostic performance is inherently limited. IMs levels vary according to the pathogen, infection type, and host characteristics[82-84]. Studies have shown that traditional IMs, such as WBC and ANC, have limited sensitivity across bacterial etiologies, often failing to reliably detect IBIs[85-86]. Even CRP shows variable performance depending on infection type and stage, with suboptimal sensitivity in infections by specific pathogens or in infants presenting early in the disease[20,26,31,69-70]. Moreover, different recommendations provide various cut-off values, further complicating clinical decision-making and limiting consistent application[12,17,52,54,52,85,87].

PCT is the most reliable IMs for identifying IBIs and has been incorporated into several clinical pathways[12,20,23,63,85,88]. Its use with other IMs, especially ANC, increases the sensitivity in ruling out an IBI, although with low specificity[12,34]. It is also linked to reduced LP rates and better risk stratification[17,52,85,87]. However, PCT does not achieve perfect discrimination and may lead to misclassification, especially when interpreted outside the clinical and temporal context, and in neonates[1,23,26,31,86]. Moreover, IMs performance varies according to the causative organism or type of IBI[86]. Increases in WBC, ANC, and CRP are lower in isolated bacteremia than in UTIs and are influenced by illness duration, and pathogen[86].

Finally, in infants ≤21 days old, PCT, CRP, and ANC can not rule out IBI, and hospital admission with empirical antibiotic therapy is still recommended regardless of clinical or laboratory findings [1,6,12,20,52,89].

In conclusion, conventional IMs mainly reflect inflammation, not its cause, and can not distinguish bacterial from viral infections, limiting their diagnostic utility[67,90]. This is a fundamental barrier to the improvement of precision medicine.

1.3.4 The lack of temporal variable integration

A critical limitation of current diagnostic methods is the lack of temporal variable integration. Most clinical algorithms use static IMs thresholds, ignoring the timing relative to symptom onset. The

host inflammatory response evolves, and early-stage IMs levels may be falsely reassuring.[20,26,31,51-52,68-70,82,84].

Recent studies have shown that the diagnostic performance of common IMs, particularly CRP and ANC, is significantly reduced in infants presenting within the first hours of fever[20,31,51,68-70]. Even PCT, though more reliable, may be influenced by timing and the biological kinetics of the specific infection[20,26,31,51,67-70,86,88]. Moreover, infants afebrile at PED presentation but with a history of fever have similar rates of BM to febrile infants and should be managed identically, complicating the accurate determination of the time from fever onset[89].

These findings highlight a mismatch between the dynamic nature of infectious diseases and the static use of diagnostic tools, suggesting that the time from fever onset should be explicitly incorporated into risk stratification models.

1.3.5 Availability and cost of biomarkers

Many algorithms rely on PCT, which is not universally available and may have higher costs than other IMs, limiting its routine use[10-11,23,62,85,91]. However, while PCT costs more than CRP, its specificity reduces unnecessary interventions and overall healthcare costs, making PCT-based approaches the most cost-effective strategies[22,91-92]. Recent approaches have focused on refining risk stratification in febrile young infants by integrating clinical variables and available laboratory markers without PCT, aiming to identify low-risk patients safely[11,93]. Some strategies remain conservative, prioritizing sensitivity and recommending broad treatment for suspected sepsis, particularly in infants under 3 months[30,54]. Moreover, the recently modified Philadelphia criteria show diagnostic performance comparable to modern algorithms, supporting their use in well-appearing infants based on clinical and basic laboratory parameters. As they do not rely on PCT, they may be a more broadly applicable option for risk stratification when advanced biomarkers are unavailable[10,62].

1.3.6 The marginal role of virology

Despite the predominance of viral infections, virological testing is inconsistently integrated into clinical decision-making, and it is unclear how viral tests should be incorporated into risk stratification recommendations[94-97].

Rapid molecular tests are becoming increasingly available. However, their use varies widely and often does not directly influence the management strategies. Studies have shown that viral testing may reduce ancillary testing, antibiotic use, and length of stay, especially during influenza season or

when incorporated into structured pathways, but it is still inconsistently used in routine febrile infant management[3,9,41,96,98-100]. Likewise, more systematic approaches based on testing multiple body sites have increased diagnostic yield for hPeV and better characterized its true epidemiology in young febrile infants[37-39,98,101].

This limited integration is relevant given the growing evidence that viral epidemiology may provide important contextual information. Viral infection has been associated with a lower risk of concomitant bacterial infection in some studies, although not sufficient to completely rule it out[9,28,41,43,94-95,102-104]. Additionally, certain viral pathogens have age- and pathogen-specific phenotypes that may refine bedside reasoning. hPeV-3 is strongly linked to sepsis-like disease in infants <3 months, often with absent CSF pleocytosis and characteristic brain Magnetic Resonance (MRI) findings[37-39,47,101,105-108]. Influenza significantly burdens infants <6 months of age and is a key cause of hospitalization during seasonal peaks. SARS-CoV-2 positivity correlates with lower UTI, bacteremia, and BM prevalence, especially in older febrile infants and those with normal IMs, though the risk is not zero.[3,9,41,44,48,50,103]. **Figure 1** is reproduced from Reference[27] and reports seasonal trends comparing viral and bacterial infections.

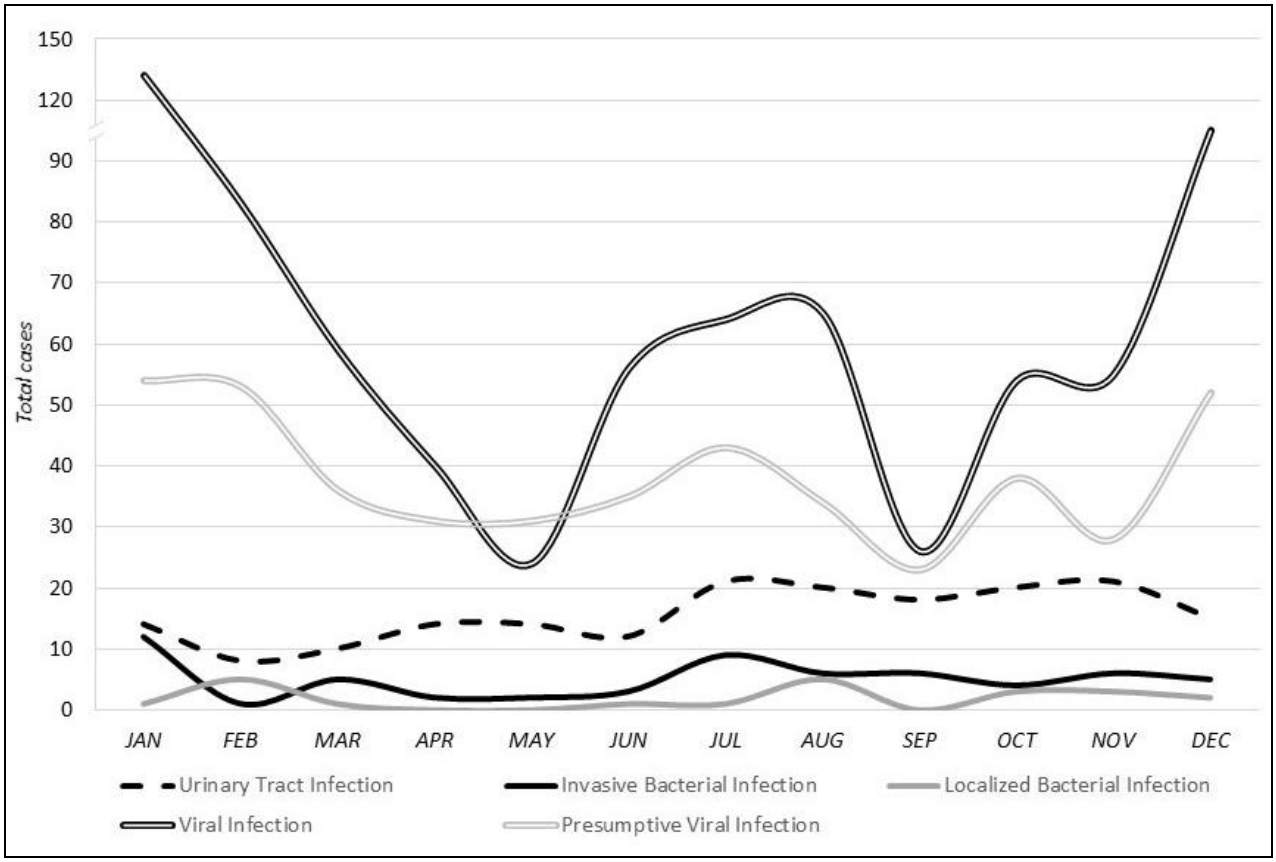

Figure 1. Seasonality of viral and bacterial infections. Monthly distribution of etiologic categories in febrile infants <90 days, showing bacterial infections (urinary tract infection, invasive bacterial infection, and localized bacterial infection) and viral etiologies (documented and presumptive). Reproduced from Reference[27].

Overall, current clinical pathways focus on excluding bacterial infections rather than identifying viral etiologies, missing an opportunity to improve diagnostic precision.

1.3.7 Urinary Tract Infections

UTIs are the most common SBI in febrile infants, accounting for approximately 90% of all SBIs in those ≤60 days of age, with an overall prevalence ranging from 4% to 20% in infants younger than 3 months[20,23,25,36,59]. Therefore, urinalysis is a cornerstone of the initial evaluation, being a readily available, rapid, and reliable screening tool with high sensitivity and specificity. Notably, urine dipstick testing alone shows an excellent negative predictive value of >98%[36,60]. Accordingly, dipstick testing may serve as an adequate stand-alone screening method while awaiting urine culture confirmation[21,36]. Despite their generally favorable prognosis, UTIs in young infants are not entirely benign diseases. Bacteremia may coexist in approximately 4–10% of febrile infants with UTI, raising concern for potential hematogenous dissemination, including CNS involvement[25]. However, evidence suggests that concomitant BM is rare, particularly among well-appearing infants aged 29–60 days[61]. Similarly, meta-analytic data indicate that well-appearing infants with positive urinalysis results are not at increased risk of BM compared with those with negative urinalysis, questioning the usefulness of routine LP in these cases[109]. Consequently, although many traditional guidelines have recommended LP with a positive urinalysis to exclude BM, this approach is controversial, especially in infants over 28 days, and current evidence supports a more individualized, risk-based strategy[59,109-110]. Specifically, LP may be avoided in well-appearing infants meeting low-risk criteria, while case-by-case assessments are warranted in higher-risk presentations[59].

Finally, clinically significant UTIs are uncommon in febrile infants without risk factors and negative dipstick results. Routine urine cultures may be unnecessary and should be reserved for those with clinical or laboratory indicators of SBI, reducing unnecessary procedures, overtesting, and ambiguous results[21,25,59,61,109-110]. Supporting this approach, multicenter data confirm that dipstick testing performs comparably to full urinalysis, reinforcing its role as an efficient screening tool60. Thus, given the high prevalence of UTIs in febrile infants, all major algorithms consistently include urinalysis as a fundamental component of the diagnostic approach, integrating it with clinical assessment and, when available, IMs to guide risk stratification and management[12,20-21,23,25,59,61,109-110].

1.3.8 Post-immunization fever

Evidence shows a lower prevalence of SBI in recently immunized infants than in non-recently immunized peers, with IBI being rare or absent. Reported SBI rates range from 2.8% (decreasing to 0.6% within 24 hours) to 3.5–5%, mostly represented by UTIs[111-113]. Thus, urine testing should be considered even within the first 24 hour[s111-112].

The risk of IBI in vaccinated infants aged 6–12 weeks is low, especially within 24 hours post-immunization[112]. Thus, post-immunization fever in young infants does not identify a high-risk subgroup for IBI. Infants presenting beyond 24–48 hours post-immunization should be managed like non-recently immunized infants, with clinical vigilance when the infant appears unwell, as IMs and leukocytosis may be misleading due to vaccine-induced immune activation[111,113-115]. Current evidence is limited, as recently immunized infants are often excluded from guideline validation studies, supporting a cautious, individualized approach based on clinical assessment, with selective investigations and shared decision-making with families[12,112-114].

1.4 Toward a new paradigm: host response, timing, and epidemiology

The limitations of the current approaches suggest the need for a paradigm shift in the evaluation and management of febrile infants. Analyzing host expression patterns in response to infections may offer an alternative diagnostic approach to classify febrile infants by infection type[68,116]. Instead of focusing solely on identifying bacterial infection, a comprehensive strategy should characterize the host immune response and integrate this information with temporal and epidemiological context[15,82-84,117-118]. Host-response–based biomarkers are a promising solution[82,84,117]. Transcriptomic approaches, especially those using type I interferon (INF) signatures, show high accuracy in distinguishing viral from bacterial infections, even in the early stages of illness[15,67-68,116-123]. These findings suggest the host immune response may offer a more direct and timely reflection of disease etiology than traditional IMs[82-84,117,124].

However, despite their biological accuracy, transcriptomic techniques are difficult to implement in clinical settings[12,67,117,124]. High costs, technical complexity, and slow turnaround limit their scalability and applicability in emergency care[67,119,123]. Thus, an ideal biomarker should be rapid, inexpensive, and applicable at the point of care, maintaining enough sensitivity to exclude or confirm bacterial infection[9]. Achieving this balance is a major challenge in the field of pediatric infectious diseases. In rthis context, attention has shifted to pragmatic transcriptomic biomarkers capturing key host response elements[84,117,124]. Flow cytometry (FC) for analyzing the immune

response in infectious diseases is gaining clinical relevance, determining leukocyte surface proteins' presence or absence[125].

Surface markers like neutrophil CD64 and monocytic CD169 are promising candidates, reflecting bacterial and interferon-mediated immune activation, respectively[123,126-129]. These markers can be measured quickly on small blood volumes and may bridge high-dimensional transcriptomic profiling and bedside clinical decision-making[117,119,126]. Recently, it was shown that during bacterial infection, IFNγ induces CD64 expression in 6 hours, while during viral infection, IFNα induces CD169 expression in under 8 hours[128-131]. Moreover, both biomarkers are independently activated by IFN, preventing false results from interactions, and are less affected by non-infectious hyperinflammatory factors, such as trauma or surgery[129-130]. Particularly, CD169, also known as sialoadhesin or Siglec-1, has been considered as an early biomarker for evaluating immune dysfunction and respiratory outcomes in coronavirus disease 2019 (COVID-19) patients[126,131-133].

2. AIM AND SCOPE

Based on these considerations, as well as on findings derived from our previous studies, this doctoral research is grounded on the hypothesis that integrating three key dimensions — *host immune response, timing of disease onset, and viral epidemiology* — may significantly enhance the accuracy and clinical applicability of current management strategies for febrile infants[26-27,38-39,42,49-50,67].

The primary objective was to determine whether the host immune response, specifically assessed through CD64 and CD169 expression following our encouraging previous interferon signature profiling results, represents a reliable tool for the early differentiation between bacterial and viral infections[67]. Building on this, the secondary aim of this study was to evaluate whether the integration of temporal variables, such as timing from fever onset and viral epidemiological context, into existing clinical algorithms can further improve risk stratification and diagnostic performance.

Ultimately, this research seeks to move beyond a static, bacterium-centered paradigm toward a dynamic, host-response–guided framework, with the goal of improving diagnostic precision, reducing unnecessary invasive procedures and treatments, and optimizing clinical outcomes in febrile infants.

3. MATERIALS AND METHODS

3.1 Overall study design

This doctoral thesis is based on a research program that integrates retrospective and prospective studies of febrile infants aged ≤90 days. This study aimed to investigate the diagnostic performance and clinical applicability of novel host-response biomarkers, focusing on the differentiation of bacterial and viral infections.

The research program was structured into four main components.

1. Evaluation of timing-dependent performance of IMs[26]
2. Retrospective epidemiological and virological analyses[27,38-39,49-50]
3. Prospective assessment of INF signature-based host-response[67]
4. Development and validation of tralational biomarkers (CD64 and CD169)

All study components were conducted within the same institutional setting and population, allowing for methodological consistency and the integration of findings. Detailed *Materials and Methods* for each individual study are reported in the respective original manuscripts included in the appendix section. Hereafter, only the characteristics of the study described in point 4 are presented.

3.2 Study setting and population

This was an observational, prospective, single-center, non-profit study. The study was conducted at the Pediatric Emergency Department (PED) of IRCCS Istituto Giannina Gaslini, a tertiary-level pediatric hospital in Genoa, Italy, with approximately 38,000 annual PED visits. The study population included infants aged ≤90 days presenting with fever, defined as axillary temperature ≥37.5°C or rectal temperature ≥38°C, either documented at home within 12h prior to PED arrival or confirmed at presentation. Infants were excluded if:

- fever was only subjectively evaluated solely by touch
- fever occurred within 48 hours following immunization
- a clear non-infectious cause of fever was identified
- they had received antibiotics in the 48h preceding PED presentation
- urinalysis was not obtained
- at least one measurement of WBC, ANC, CRP, PCT was not obtained
- were preterm < 37 week of gestational age at birth
- a parent or guardian refused consent

The data collected included patient age, gestational age, gender, past medical history, and duration of fever. Standard laboratory investigations were performed on febrile infants.

3.2.1. Healthy control group

Control samples were obtained from age-matched infants presenting to the PED with non-infectious conditions, such as minor trauma or surgical complaints, with no clinical or laboratory evidence of infection at the time of evaluation.

3.3 Laboratory procedures

The standard diagnostic workup included complete blood count, CRP, PCT, urine analysis and cultures, blood cultures, and CSF analysis when clinically indicated. Other microbiological and molecular tests were performed according to institutional protocols.

3.3.1 Leukocyte subpopulation identification

Leukocyte subpopulations were identified on whole blood samples using a standard gating strategy based on forward scatter area (FSC-A) and side scatter area (SSC-A) dot plots. FSC-A reflects cell size, whereas SSC-A reflects internal granularity and cytoplasmic complexity. This approach allows the discrimination of three distinct populations based on physical parameters alone, prior to fluorescence analysis. The gating strategy is shown in Figure 2. Both leukocyte subpopulation identification and subsequent CD64 and CD169 expression analyses were performed on residual blood obtained from ethylene-diamine-tetra-acetic acid (EDTA) tubes collected for routine blood count testing, without additional blood sampling.

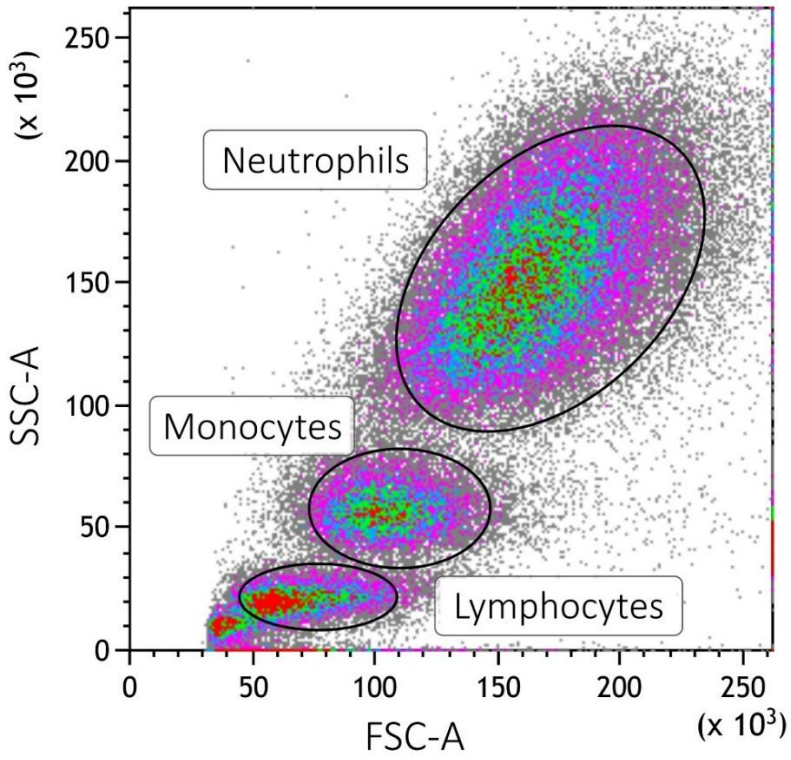

Figure 2. Gating strategy for leukocyte subpopulation identification. Representative FSC-A (forward scatter area, index of cell size) vs SSC-A (side scatter area, index of cell granularity and internal complexity) dot plot of whole blood samples, illustrating the gating strategy used to identify neutrophils, monocytes, and lymphocytes prior to the analysis of CD64 and CD169 surface expression. Three distinct populations were identified based on physical parameters alone: lymphocytes (small, low-granularity cells, lower left gate), monocytes (intermediate size and granularity, central gate), and neutrophils (large, highly granular cells owing to their multilobed nucleus, upper right gate). Overlapping colors represent individual samples from patients with bacterial infection, non-bacterial infection, and healthy controls, demonstrating the reproducibility of the gating strategy across study participants.

3.3.2 CD64 and CD169 analysis

Aliquots of 100 µL of blood were incubated with a myeloid activation antibody panel. After erythrocyte lysis, the expression of CD64 on neutrophils and CD169 on monocytes was quantified as the geometric mean fluorescence intensity (GeoMFI), normalized to lymphocyte expression within the same sample (CD64 neutrophil/CD64 lymphocyte and CD169 monocyte/CD169 lymphocyte, respectively). Hereafter, CD64 neutrophil/CD64 lymphocyte is referred to as the CD64 ratio, and CD169 monocyte/CD169 lymphocyte as the CD169 ratio. Positivity thresholds were defined as CD64 ratio >8 and CD169 ratio >4, based on prior studies and established laboratory standards[126,130]. These biomarkers were evaluated individually and in combination, including the CD64/CD169 combined ratio, defined as [(CD64 neutrophil/CD64 lymphocyte) / (CD169 monocyte/CD169 lymphocyte)]. Laboratory personnel performing CD64 and CD169 analyses were blinded to the patients' clinical data and final diagnosis.

Representative flow cytometry histograms illustrating the separation between monocyte/neutrophil and lymphocyte fluorescence peaks in healthy controls (HC) and patients, and the principle underlying the GeoMFI normalization approach, are shown in **Figure 3**.

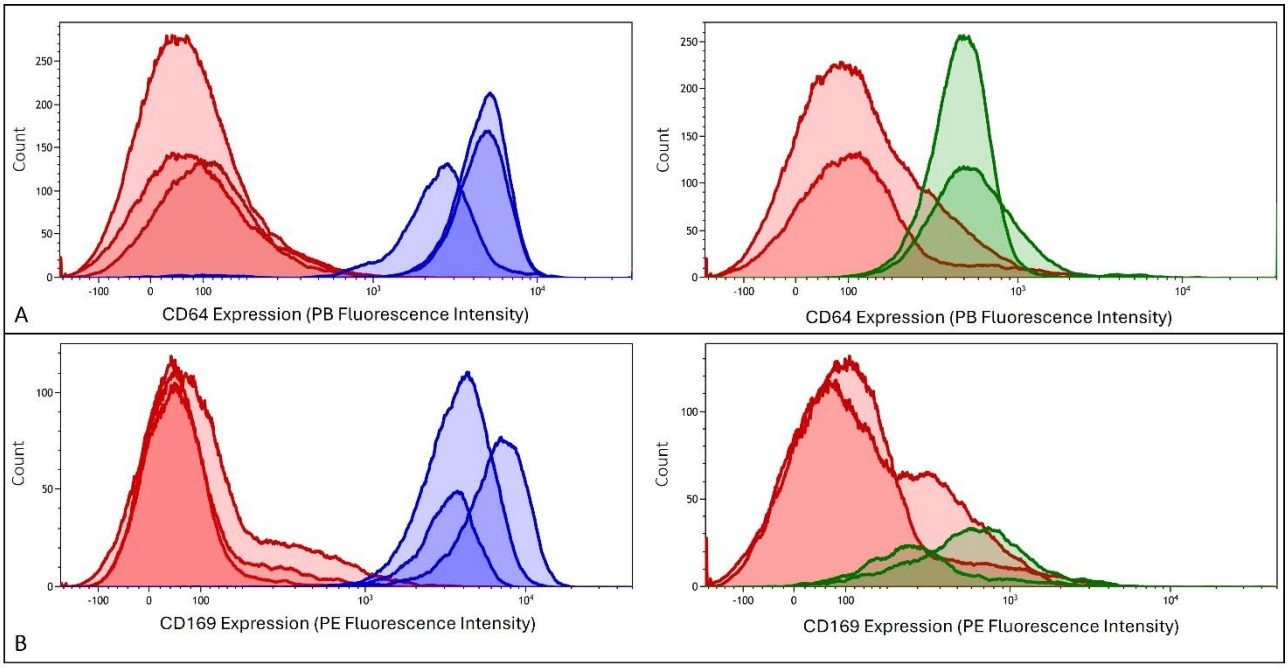

Figure 3. Representative flow cytometry histograms illustrating the CD169 and CD64 expression ratios in healthy controls and patients. Each histogram represents two cellular populations from the same blood sample: the cell of interest (monocytes for CD169, blue; neutrophils for CD64, green) and autologous lymphocytes (red), which serve as an internal negative reference given their constitutive absence of CD169 and CD64 expression. The x-axis represents the fluorescence intensity of the indicated marker (CD169-PE or CD64-PB) displayed on a logarithmic scale, reflecting the level of surface expression per cell. The y-axis represents the cell count, depicting the distribution of marker expression across each gated population. Representative histograms were generated from three individual samples for each infectious group (bacterial and non-bacterial infections) and two samples for the healthy control group for both CD64 and CD169 analyses. **Panel A (CD64, neutrophils):** In healthy controls (right subpanel, green), neutrophil CD64 peaks were modestly right-shifted relative to lymphocytes, consistent with low baseline CD64 expression. In patients with bacterial infection (left subpanel, blue), neutrophil CD64 peaks were strongly right-shifted, reflecting IFN-γ-mediated upregulation and a high neutrophil/lymphocyte CD64 ratio. **Panel B (CD169, monocytes):** In healthy controls (right subpanel, green), the monocyte and lymphocyte CD169 fluorescence peaks overlap substantially, reflecting a low baseline CD169 expression and a ratio close to unity. In patients with non-bacterial infection (left subpanel, blue), monocyte CD169 peaks were markedly right-shifted relative to lymphocytes, reflecting IFN-α–mediated CD169 upregulation and a high monocyte/lymphocyte CD169 ratio. *PB: Pacific Blue (fluorochrome used for CD64 detection); PE: phycoerythrin (fluorochrome used for CD169 detection).*

3.4 Study definitions and outcome measures

Bacteremia and BM were defined as the growth of a single pathogen in the blood or CSF, respectively. UTI was defined as the growth of a single pathogen in the urine with colony counts (colony-forming units, CFUs) meeting the criteria of ≥100.000 CFUs/mL, in association with an abnormal dipstick test or urinalysis (positive for leukocytes, leukocyte esterase, or nitrites). Blood and CSF cultures were considered to reflect the growth of contaminants when bacterial isolates were not commonly accepted as pathogens. We defined SBI as the presence of BM, bacteremia, or UTI and IBI as the presence of BM or bacteremia.

Patients were classified as having bacterial infections ("BI" group) if they turned out to have a SBI, otherwise, they were assigned to the non-bacterial infection ("non-BI") group. To detect viral etiological agents, multiplex real-time polymerase chain reaction (RT-PCR) assays or rapid antigen tests were performed on nasopharyngeal samples or CSF if an LP was performed. Patients who did not undergo culture testing were classified as non-BI if they exhibited a favorable, self-limiting clinical course with spontaneous defervescence during at least 24h of in-hospital monitoring without antibiotic administration, even if a viral etiology was not ultimately identified. Patients in whom no pathogen was identified were classified as non-BI if all cultures performed prior to antibiotic administration were negative or if they experienced a favorable and self-limiting course with spontaneous defervescence and recovery during hospitalization without antibiotic therapy.

The enrolled patients were categorized into these two groups by PED pediatricians blinded to the CD64 and CD169 results.

3.5 Statistical analysis

Demographic data, laboratory findings, and CD64 and CD169 results were compared between the BI, non-BI, and HC groups. The data were organized in a tabular form and analyzed anonymously. Continuous variables are reported as medians and interquartile ranges (IQR), and categorical variables are reported as frequencies and percentages. Comparisons between categorical variables were performed using cross-tabulations, and statistical significance was assessed using the two-sided Fisher's exact test. For comparison of continuous variables between the two groups, parametric (two-sided Student's t-test) or non-parametric (two-sided Mann-Whitney U test) tests were used based on the data distribution and sample size. For comparisons of multiple independent variables, the Kruskal-Wallis test was employed, followed by Dunn's test for pairwise

multiple comparisons. Multiple tests were controlled using the Benjamini–Hochberg correction, where appropriate. Statistical significance was set at a two-sided $p < 0.05$.

Receiver operating characteristic (ROC) curves were used to evaluate the diagnostic performance, including the analysis of single biomarkers and combined models. A logistic regression model was employed to assess predictive performance, with model discrimination evaluated by the area under the ROC curve (AUC). In addition to the predefined analytical thresholds, data-driven optimal cutoff values for each biomarker were derived using ROC curve analysis. The optimal threshold was identified by maximizing the Youden index (sensitivity + specificity − 1). For CD169, which reflects an interferon-mediated viral response, inverse-direction ROC analysis was applied, considering lower values indicative of bacterial infection. All statistical analyses were performed using GraphPad Prism version 9.1.0 for Windows (GraphPad Software, San Diego, California, www.graphpad.com).

3.6 Ethical considerations

The study was approved by the local Ethics Committee (CET – Liguria; approval number: 262/2024). For prospective components, informed consent was obtained from the parents or legal guardians. Retrospective analyses were performed using anonymized data.

4. RESULTS

4.1 Overview

Detailed results regarding (1) the evaluation of timing-dependent performance of IMs, (2) retrospective epidemiological and virological analyses, and (3) the prospective assessment of interferon signature–based host response are reported in the respective original manuscripts included in the Appendix section[26-27,38-39,49-50,67]. Hereafter, only the results of the preliminary diagnostic performance of the membrane biomarkers CD64 and CD169 are reported.

Sixty-one infants were included, of whom 63% were male. A total of 29 (47.5%) patients were enrolled in the non-BI group, 12 (19.7%) in the BI group, and 20 (32.8%) in the HC group. The median age was 52 days (IQR 25–65) in the non-BI group, 36 days (32–48) in the BI Group, and 28 days (21–44) in the HC group, with no significant difference across groups. Similarly, the interval between fever onset and blood sampling did not differ significantly between the infectious groups, with median values of 18 hours (IQR 6–24) in the non-BI group and 21 hours (6–27) in the BI group.

4.2 Preliminary diagnostic performance of CD64 and CD169

The non-BI group displayed a CD169-predominant profile, whereas the BI group showed a CD64-predominant pattern. The HC group was characterized by a low baseline expression of both biomarkers. In the three-group comparison, host response biomarkers and their derived ratios showed the strongest discriminatory performance. The CD64 ratio was 10.57 (IQR 8.02–14.88) in the non-BI group and 39.46 (16.08–42.48) in the BI group. The CD169 ratio was 26.62 (17.15–47.73) in the non-BI group and 2.94 (2.40–3.90) in the BI group. The CD64/CD169 combined ratio was 0.34 (0.23–0.60) in the non-BI group and 12.99 (5.72–15.05) in the BI group. All three between-group comparisons were statistically significant ($p<0.001$). The HC group was characterized by low baseline expression of both markers: CD64 ratio 6.44 (5.00–8.12), CD169 ratio 3.02 (2.65–4.07), and CD64/CD169 ratio 1.98 (1.10–2.55). **Figure 4** shows the findings regarding the CD64 and CD169 ratios. **Figure 5** shows the distribution of the CD64/CD169 ratio in the three study groups. The absolute fluorescence values confirmed these findings. Neutrophil CD64 expression was significantly higher in the BI group than in the non-BI and HC groups ($p<0.001$), whereas monocyte CD169 expression was markedly elevated in the non-BI group than in the other groups ($p<0.001$).

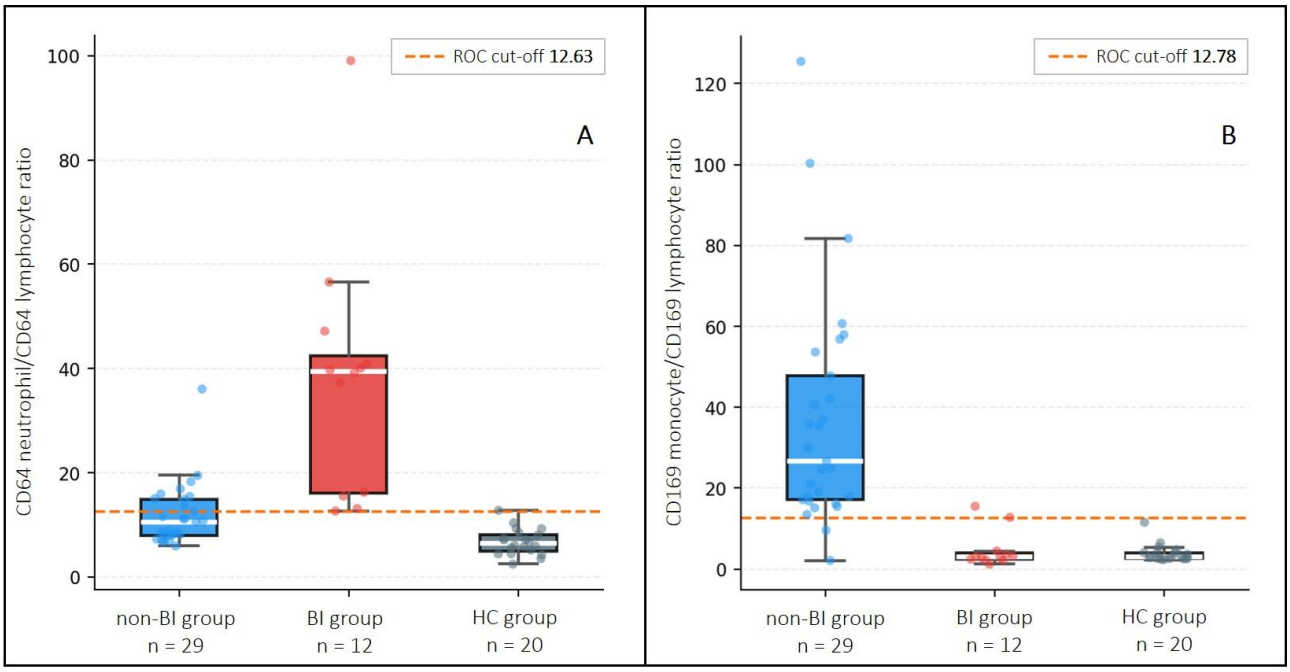

Figure 4. Neutrophil CD64 and Monocyte CD169 expression across the study groups. (A) Distribution of the CD64 neutrophil/CD64 lymphocyte ratio and **(B)** Distribution of the CD169 monocyte/CD169 lymphocyte ratio across the three study groups. Data are presented as boxplots, indicating the median and interquartile range. Statistical comparisons across groups were performed using the Kruskal–Wallis test, which showed a highly significant difference ($p < 0.001$). Dashed lines indicate the ROC-derived optimal cutoff values. *BI: bacterial infection group; HC: healthy control group; non-BI: non-bacterial infection group; ROC: receiver operating characteristic.*

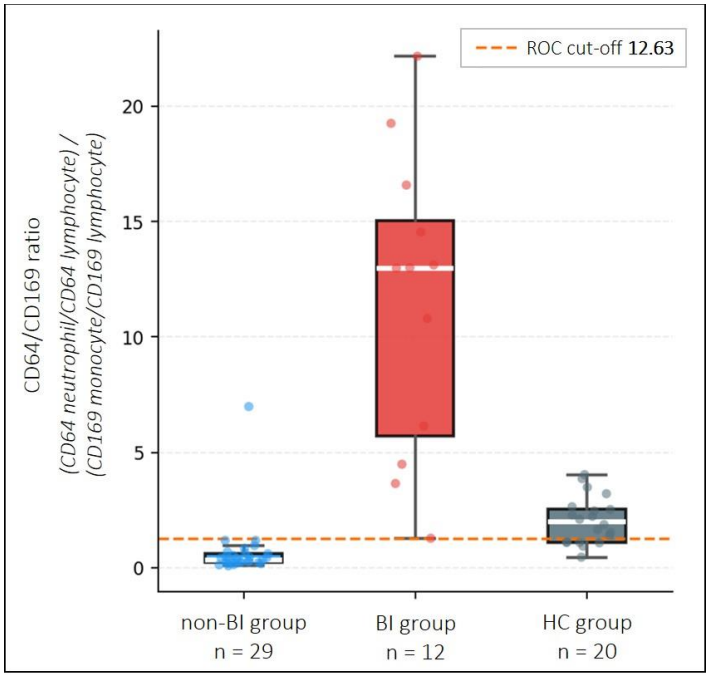

Figure 5. Distribution of CD64/CD169 ratio in the study groups. Data are shown as box plots (median and interquartile range) with individual data points. Statistical comparison using the Kruskal–Wallis test showed a highly significant

difference between the groups (p < 0.001). Dashed lines indicate the ROC-derived optimal cutoff values. *BI: bacterial infection group; HC: healthy control group; non-BI: non-bacterial infection group; ROC: receiver operating characteristic.*

4.3 Conventional inflammatory biomarkers

Both CRP and PCT levels were significantly higher in the BI group than in the non-BI group. CRP was 0.67 mg/dL (IQR 0.00–2.39) in the non-BI group and 7.90 mg/dL (IQR 5.56–11.41) in the BI group (p<0.001). Similarly, PCT was 0.15 ng/mL (IQR 0.13–0.20) in the non-BI group and 1.49 ng/mL (IQR 0.74–2.58) in the BI group (p<0.001). WBC and ANC were also higher in the BI group than in the non-BI group (p = 0.012 and p = 0.005, respectively). Lymphocyte, monocyte, and platelet counts were not significantly associated with the infection type. Table 2 reports the main findings of the present research.

	Total *n = 61*	**Non-BI** *n = 29*	**BI group** *n = 12*	**HC group** *n = 20*	***p***
Age *days (IQR)*	36 (22-59)	52 (25–65)	36 (32–48)	28 (21–44)	0.21
Gender *male (%)*	39 (63)	16 (55)	10 (83)	13 (65)	0.23
Fever onset to blood sampling *hours (IQR)*	18 (6-24)	18 (6–24)	21 (6–27)	—	0.54
WBC (/mm³) *(median, IQR)*	10130 (8040-12280)	9510 (7230–11190)	11990 (10322–21172)	—	**0.012**
Neutrophils (/mm³) *(median, IQR)*	4970 (3520-5810)	4320 (2510–5430)	6165 (4392–10508)	—	**0.005**
Lymphocytes (/mm³) *(median, IQR)*	3860 (2530-5360)	3720 (2430–5360)	4835 (3215–5370)	—	0.30
Monocytes (/mm³) *(median, IQR)*	990 (530-1450)	860 (520–1320)	1165 (852–2668)	—	0.13
Platelets (/mm³) *(median, IQR)*	376000 (327000-510000)	362000 (327000–509000)	402000 (331000–491500)	—	0.42
CRP (mg/dL) *(median, IQR)*	1.44 (0.00-4.50)	0.67 (0.00–2.39)	7.90 (5.56–11.41)	—	**<0.001**
PCT (ng/mL) *(median, IQR)*	0.20 (0.14-0.81)	0.15 (0.13–0.20)	1.49 (0.74–2.58)	—	**<0.001**
CD64 ratio *(median, IQR)*	9.26 (7.11-15.45)	10.57 (8.02–14.88)	39.46 (16.08–42.48)	6.44 (5.00–8.12)	**<0.001**
CD169 ratio *(median, IQR)*	9.59 (2.82-24.81)	26.62 (17.15–47.73)	2.94 (2.40–3.90)	3.02 (2.65–4.07)	**<0.001**
CD64/CD169 ratio *(median, IQR)*	1.10 (0.42-3.20)	0.34 (0.23–0.60)	12.99 (5.72–15.05)	1.98 (1.10–2.55)	**<0.001**

Table 2. Clinical and laboratory characteristics of the study population. Values are reported as medians (interquartile range). P-values refer to comparisons between the non-BI and BI groups using the Mann–Whitney U test. For variables available across all three groups, overall comparisons were performed using the Kruskal–Wallis test. *BI: bacterial*

infection group; CRP: C-reactive protein; HC: healthy control group; non-BI: non-bacterial infection group; PCT: procalcitonin; WBC: white blood cell count.

4.4 ROC analysis

ROC analysis confirmed the superior diagnostic performance of the host response biomarkers. The CD64/CD169 ratio achieved the highest discrimination between the BI and non-BI groups, with an AUC of 0.989 (sensitivity 100%, specificity 96.6%, optimal cut-off ≥1.27). Among the single biomarkers, the CD64 ratio showed an AUC of 0.922 (sensitivity 100%, specificity 69.0%), whereas PCT and CRP had AUCs of approximately 0.94 and 0.90, respectively. As expected from its biological role, CD169 alone showed inverse-direction discrimination with an AUC of 0.954 (sensitivity, 91.7%; specificity, 93.1%). Data-driven optimal thresholds identified a CD64 ratio of approximately ≥12.63 (Youden index) and a CD169 ratio of approximately ≤12.78 (inverse direction, Youden index) as the best discriminating values for bacterial infection. **Figure 6** shows a scatter plot of CD64 and CD169 with ROC-derived cut-offs. Multivariable models combining CD64, CD169, and PCT showed only marginal improvement over the CD64/CD169 ratio alone, suggesting that the combined host response signature captured most of the relevant diagnostic information. **Figure 7** shows the ROC curves for the single biomarkers and combined models.

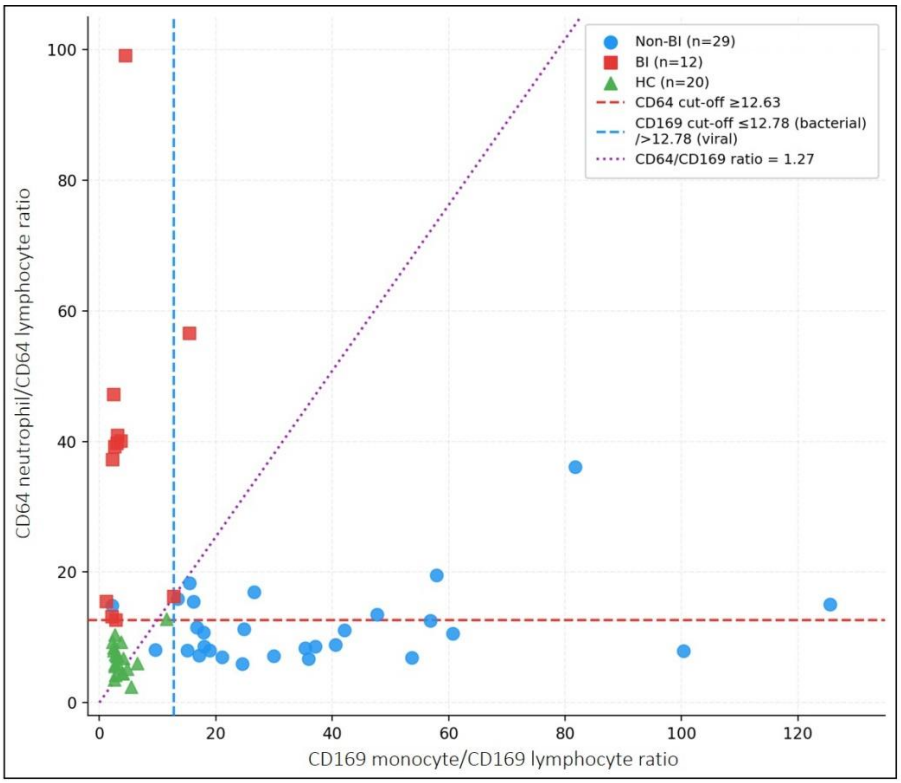

Figure 6. Scatter plot of CD169 monocyte / CD169 lymphocyte ratio (x-axis) versus CD64 neutrophil / CD64 lymphocyte ratio (y-axis) for all study participants. Each point represents an individual patient stratified by the diagnostic group.

Dashed lines indicate the ROC-derived optimal thresholds for bacterial infection (CD169 ≈ 12.78; CD64 ≈ 12.63), calculated using the Youden index. These thresholds correspond to statistically significant discrimination between the non-BI and BI groups ($p < 0.001$, Mann–Whitney U test). *BI: bacterial infection group; HC: healthy control group; non-BI: non-bacterial infection group.*

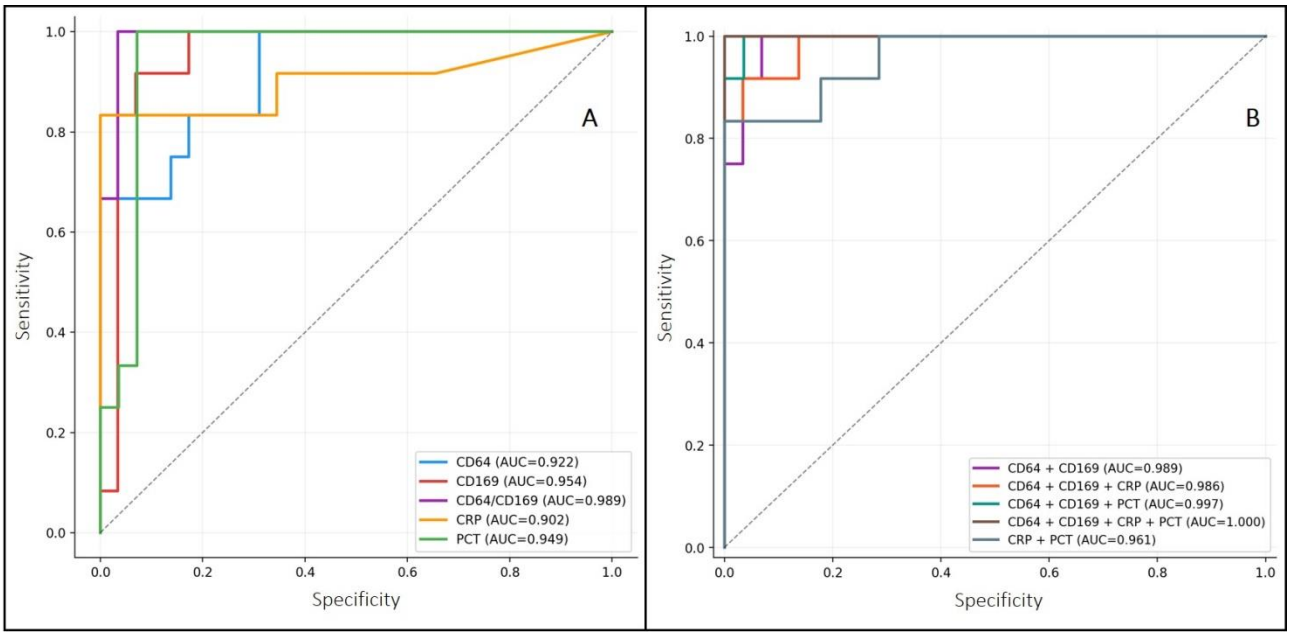

Figure 7. A. ROC curves evaluating the diagnostic performance of individual biomarkers for discrimination between the BI and non-BI groups. The CD64/CD169 ratio had the highest area under the curve (AUC = 0.989), followed by CD64 (AUC ≈ 0.922), PCT (AUC ≈ 0.949), and CRP (AUC ≈ 0.902). CD169 alone showed inverse-direction discrimination, reflecting its role as a marker of viral response. The statistical significance of group separation for all biomarkers was confirmed using the Mann–Whitney U test (all $p < 0.001$). **B. ROC curves for multivariate logistic regression models combining CD64, CD169, and conventional inflammatory markers.** Models including CD64 and CD169 showed excellent discriminative performance, with only marginal improvement observed after the addition of PCT or CRP. These findings suggest that the combined host response signature captures most of the relevant diagnostic information. Model performance was evaluated using AUC, and all variables were significantly associated with infection status ($p<0.001$ in univariable analyses). *CRP: C-reactive protein; PCT: procalcitonin.*

5. DISCUSSION

5.1 Main findings

The diagnostic evaluation of febrile infants younger than 90 days remains safe but is intrinsically imprecise[58]. Balancing the common scenario of a young febrile infant recovering uneventfully from a viral infection with the rare catastrophic possibility of an IBI should be the main aim of clinical research in this field[58]. Current management strategies effectively minimize missed SBIs but cause substantial overtreatment, as they are based on a bacteria-centered, static view of a biologically dynamic condition[12-13,20,23,54]. Taken together, the results of this research program suggest that the limitations of current management do not depend on a single weak test but on a broader conceptual issue. Specifically, current models insufficiently integrate three dimensions central to the biology and clinical behavior of fever in early infancy: *time from fever onset*, *viral epidemiology and virological heterogeneity*, and *host-response specificity*[15,52,67,82,84,90,117]. Therefore, this thesis supports the shift from a static model focused on excluding bacterial infections to an integrated framework in which inflammatory response, temporal kinetics, and virological context are interpreted together. Within these results, the thesis provides four main contributions.

First, it shows that conventional IMs have important time-dependent limitations, particularly in the earliest hours of fever[20,26,31,68-69].

Second, it demonstrates that viral infections are not merely common secondary diagnoses, but dominant and clinically heterogeneous etiologies with major implications for risk stratification[3,9,28-29,38-39,41-42,50,94-95,98,101-103,106].

Third, it provides biological proof-of-concept that host-response approaches, particularly interferon-based signatures and host gene expression tests, outperform conventional biomarkers in distinguishing bacterial from non-bacterial infections[15,67,82-84,116-118,120-122].

Fourth, it provides preliminary translational evidence that CD64 and CD169 may represent clinically applicable surrogates for these host response pathways[126-131].

5.2 The paradox of febrile infant 2.0

A central objective of existing approaches is to develop accurate risk-stratification strategies to balance early SBI identification with avoiding unnecessary invasive procedures and treatments. This conservative management of febrile infants is therefore justified. Young age is linked to a non-negligible prevalence of SBI and IBI, clinical presentation is often nonspecific, and deterioration may be rapid[1,12-13,52,16,23]. For this reason, management strategies have traditionally maximized

sensitivity, even at the cost of specificity[2,10,12,18,20,23,35,62]. However, these approaches result in a large amount of diagnostic overreach. In the broad epidemiologic cohort, SBI represented 15% of cases, meaning that most infants evaluated through aggressive bacterial-oriented pathways did not ultimately have bacterial disease[25,27-29,102]. This has resulted in a persistent dualism between two imperatives: not missing infants with SBI while avoiding unnecessary invasive procedures in the majority with non-bacterial disease. This discrepancy has been recognized in the literature, but its implications are often underestimated.

This paradox is more striking given that viral infections are not just common but dominant, with prevalence changing across age groups, seasons, and periods.[27,94-96]. Thus, the problem is not only that many infants may be overtreated. It is that informations already available in the epidemiology and biology of underlying diseases are often underutilized.

However, the results of this study do not argue against caution. Rather, they suggest that caution should be refined and better targeted in the future.

5.3 Time matters: the limits of static biomarkers

The diagnostic performance of conventional IMs is strongly conditioned by the time from fever onset. In our retrospective study on IMs, CRP, PCT, and ANC differed significantly between infants with SBI presenting within the first 12 hours of fever and those presenting later, demonstrating that bacterial inflammatory profiles evolve substantially even within a short early window[20,27,69-70]. This is biologically plausible and consistent with the known kinetics of acute-phase proteins and myeloid-response markers. An IM measured too early may falsely reassure because the inflammatory response has not fully developed. CRP and PCT are the most commonly collected IMs, peaking at 24–36 and 8–24 h of fever, respectively[27]. Thus, they should be used carefully to stratify infectious risks, especially in children with short fevers[20,27,69-70]. Table 3, reproduced from Reference[26], shows how early tests can be negative even in SBI, falsely reassuring, and mimicking a presumptive viral, benign episode.

	All	SBI ≤ 12h fever *group n° 1*	SBI > 12h fever *group n° 2*	CBE *group n° 3*	1 vs 2	1 vs 3	2 vs 3
	n = 308	*n = 56*	*n = 34*	*n = 218*	***p***		
Gender *male (%)*	205 (66.6)	39 (69.6)	27 (79.4)	139 (63.8)	0.34	0.44	0.08
ANC *absolute values*	5081.16 ±3884.21	6336.27 ±4044.14	9318.59 ±6058.07	4097.86 ±2725.22	**0.006**	**0.0001**	**0.0001**
ANC *> 10000 el/mm^3 (%)*	34 (11.0)	10 (17.9)	13 (38.2)	11 (5.0)	**0.05**	**0.003**	**0.0001**
CRP *absolute values*	3.01±4.05	2.25±2.46	9.49±6.89	2.20±2.64	**0.0001**	0.63	**0.0001**
CRP *> 20 mg/l (%)*	138 (45.0)	23 (41.1)	29 (85.3)	86 (39.6)	**0.0001**	0.88	**0.0001**
PCT *absolute values*	2.00±5.62	1.77±3.98	7.78±12.06	0.76±1.97	**0.0001**	0.06	**0.0001**
PCT *> 0,5 ng/ml (%)*	87 (41.6)	19 (35.2)	24 (82.8)	44 (34.9)	**0.0001**	1	**0.0001**

Table 3. Comparison of inflammatory marker values between the SBI ≤ 12h fever group, SBI > 12h fever group, and viral infection, defined as the clinical benign episode (CBE) group. The three groups reported statistically significant differences in inflammatory markers. Data are described as mean and standard deviation for continuous variables and as absolute and relative frequencies for categorical variables. *ANC = absolute neutrophil count; CBE = clinically benign episode; CRP = C-reactive protein; PCT = procalcitonin; SBI = serious bacterial infection.* Adapted from Reference[26].

As expected, in this research CRP and PCT showed significant differences between the BI and non-BI groups (p<0.001 for both), consistent with their role as acute-phase reactants in bacterial infection and their incorporation into all major risk-stratification algorithms[12,20,23,63,88]. However, their discriminatory performance must be contextualized against blood sampling timing. In our cohort, both the BI and non-BI groups had a median fever-to-bloodwork interval of over 12 hours (21 and 18 hours, respectively), which corresponds to the threshold beyond which CRP and PCT levels achieve more reliable elevation, as shown in our previously published data.[26]. Although WBC and ANC values were statistically significant between the BI and non-BI groups, these findings should be interpreted with caution. Both markers have limited pathogen specificity and variable diagnostic performance across bacterial aetiologies and infection stages[49,85-86]. Furthermore, the BI group had a younger median age than the non-BI group (36 vs 52 days), and elevated leukocyte reference ranges in early life may partly explain the differences, independent of infectious aetiology[7,8,49]. Thus, they should not be considered as indicators of a greater inflammatory burden[49].

Recent evidence suggests that PCT plus CRP has high sensitivity for IBI while maintaining high specificity, even in patients <21 days old. However, the time factor still remains unknown[1,134].

This result aligns with the growing literature indicating febrile infant algorithms may be weaker in infants with short fever duration[1,20,31,51,68-70]. Our prospective interferon-signature study reinforced this point, as conventional IMs were less informative in the earliest phase of illness, whereas host-response profiling retained discriminatory value[15,67-68,82-84,116,120-122,130]. This means that a normal CRP, ANC, or even PCT obtained very early after fever onset should not be interpreted with the same confidence as identical results obtained later. This may partly explain why some infants classified as low risk still harbor SBI despite appropriate algorithm application[31,20,51].

Moreover, the time to blood and CSF positivity is shorter for bacterial pathogens than for contaminants, but still requires approximately 24 hours81. This difference can help reduce hospitalization duration and avoid unnecessary antibiotics but still requires patient admission while awaiting the report[81].

5.4 The central role of viral infections

Another major conclusion of this research is that virology should shift from a secondary to a central role in the diagnosis of febrile infants. Current pathways focus on ruling out bacterial diseases, while viral causes are considered optional contextual information. This approach is incomplete because prompt viral identification may reduce the need for lumbar puncture, hospitalizations, and unnecessary treatment of neonates or infants failing initial low-risk criteria.[97].

The results demonstrated that viral infections predominated across all age groups and displayed marked seasonality, whereas bacterial infections remained comparatively stable and non-seasonal[27]. This distinction is clinically meaningful because knowing viral epidemiology may help in differential diagnosis[9,27,41]. Respiratory viruses are dominant in winter. In summer and early autumn, EV and hPeV become more relevant[3,9,27,41,44,98,135-136]. SARS-CoV-2 apparently shows both winter and spring-summer peak[9,41,103-104]. Such contextual information is too important to be marginal. Viral etiologies range from mild self-limited syndromes to severe sepsis-like, neurologic, and inflammatory disease[9,37-39,41-42,47,50,98,101,106,108,135]. Recognizing this heterogeneity is essential for integrating virology into clinical decision-making, allowing reduced antibiotic treatment duration and early discharge[3,96-97]. Moreover, a viral etiological diagnosis can allow for targeted therapy, such as oseltamivir in rare severe influenza infections and remdesivir in SARS-CoV-2 infection, which appear safe and effective in this age group, or anakinra in cases of HLH or myocarditis during EV/hPeV infections.[38,137-142].

However, viral results should not be used simplistically because viral positivity reduces but does not eliminate the risk of bacterial infection. This has been shown repeatedly in prior studies and is confirmed by our own data, in which approximately 10% of SBI cases had documented viral co-infection[9,24,27-28,102]. Therefore, virology can not simply be used as a *de-escalation switch* and not replace guidelines and algorithms. Instead, it should be interpreted probabilistically, considering age, urinalysis, IMs, and time from fever onset.

5.5 Different viruses, different meanings

As mentioned above, the overall risk of co-infection is approximately 5-10% with a non-negligible risk of SBIs, including bacteremia and BM[28]. However, these risks must be considered separately for each virus. The case-specific viral studies in this thesis show that viral diagnoses have varying implications. Thus, viral diagnosis in early infancy may lower bacterial suspicion, identify a severe viral phenotype, or both[94-96]. **Table 4** summarizes the principal clinical and epidemiological characteristics of the main viral pathogens in febrile infants ≤90 days old. **Figure 8**, adapted from Reference[27], reports the seasonal trends of the main viruses causing fever in infants.

Virus	Seasonality	SBI Co-infection Risk	IBI Co-infection Risk	Severe Phenotype	Key Clinical Implications
RSV [3,9,12,28,43,95-96]	Winter–Spring (Oct–Mar)	Low–moderate *UTI risk reduced vs virus-negative infants*	Low *Rare bacteraemia and meningitis*	Bronchiolitis, apnoea, respiratory failure Rarely sepsis-like	RSV excluded from some algorithms (AAP) Positive test supports conservative approach in well-appearing infants >28 days
Influenza A/B [3,41,44,49,136, 138,149,152]	Winter (Nov–Mar)	Moderate *SBI rate lower than virus-negative infants* *UTI may still occur*	Very Low *No meningitis reported in recent cohorts*	Generally mild Rarely sepsis-like or HLH	Positive RADT reduces LP, antibiotic use, and hospitalisation Oseltamivir indicated in neonates and severe cases
SARS-CoV-2 [24,50,103-104, 143-148]	Winter *Spring–Summer peaks*	Low-moderate *Significantly lower UTI vs virus-negative*	Very low *No bacteraemia reported in most series*	Generally mild Rare hyperinflammatory syndrome (older infants)	Conservative management appropriate in well-appearing infants >28 days with normal IM Remdesivir in severe cases
EV [28,38,4243,45, 105,108,142]	Summer–Autumn (Jul–Oct)	Moderate	Low *Bacteraemia rare, bacterial meningitis uncommon*	Sepsis-like illness Meningoencephalitis Myocarditis HLH	LP strongly recommended in neonates with suspected EV CSF RT-PCR is essential Anakinra considered in HLH/myocarditis
hPeV [3739,47,98,101, 105,107,141]	Summer–Autumn (Jul–Oct)	Moderate	Low *Bacterial meningitis rare but significant clinical overlap*	Sepsis-like illness Encephalitis Periventricular white matter injury	LP should be performed CSF pleocytosis often absent Brain MRI is recommended Testing multiple body sites increases diagnostic yield
hRV [43,95,102]	Year-round *Spring–Autumn peaks*	Does not reliably reduce UTI or IBI risk, particularly in neonates		Typically mild URI Rarely lower respiratory tract involvement	hRV positivity alone should not alter sepsis workup in infants ≤28 days Conservative approach in 29–90 days with normal IM
Adenovirus [153-154]	Year-round *Slight peaks in Spring and Autumn*	No specific data available in febrile infants ≤90 days		Self-limiting in most immunocompetent hosts Risk of disseminated infection in neonates	Consider in neonates with fever, hepatitis, or pneumonia without bacterial source Standard bacterial workup should not be omitted
hPIV 1–3 [3,9,24,43]	hPIV-1 and 2: Autumn–Winter hPIV-3: Spring–Summer	No specific data available in febrile infants ≤90 days		Croup (hPIV-1/2) Bronchiolitis and pneumonia (hPIV-3) Less severe than RSV	No specific guidelines for hPIV in febrile infants ≤90 days Clinical approach analogous to RSV in well-appearing infants
hMPV [3,9,43,95]	Late Winter–Spring	Low, similar to RSV *Limited specific data in ≤90 days*	Low	Bronchiolitis Lower respiratory tract infection	Generally managed similarly to RSV Conservative approach in well-appearing 29-90 days infants
HSV-1/2 [159-161]	Year-round *No seasonal variation*	Low; bacterial co-infection is rare (~3%) but reported *HSV must be considered independently in the differential, not as a modifier of bacterial risk*		Skin-eye-mouth, 41% Encephalitis, 35% (*mortality 50%, 4% with acyclovir)* Disseminated, 24% *(mortality 85%, 30% with acyclovir)*	HSV must be systematically considered in febrile neonates ≤21 days (particularly with seizures, vesicular lesions, elevated liver enzymes) CSF RT-PCR is the diagnostic gold standard Acyclovir should be initiated immediately pending CSF RT-PCR results

Table 4. Virus-specific clinical characteristics in febrile infants ≤90 days of age. The table summarizes the clinical and epidemiological features of the main viral pathogens relevant to the diagnostic evaluation of febrile infants ≤90 days. The SBI co-infection risk is categorized as very low (<1%), low (1–3%), low–moderate (3–5%), or moderate (5–10%), based on reported rates across published cohort studies and systematic reviews; estimates may vary substantially depending on the study population, season, viral prevalence, and the diagnostic approach. For Adenovirus, Human Parainfluenza Virus, and HSV, cells indicating 'No specific data available' reflect the absence of dedicated studies evaluating SBI co-infection risk specifically in febrile infants ≤90 days; standard clinical workup should not be modified solely on this basis. HSV is included as a distinct entry given its unique clinical relevance as an independent, time-critical diagnosis requiring empirical antiviral therapy rather than as a modifier of bacterial co-infection risk. *AAP: American Academy of Pediatrics; CNS: central nervous system; CSF: cerebrospinal fluid; EV: enterovirus; HLH: haemophagocytic lymphohistiocytosis; hPeV: human parechovirus; hMPV: human metapneumovirus; hPIV: human parainfluenza virus: hRV: human rhinovirus; HSV: herpes simplex virus; IBI: invasive bacterial infection (bacteremia or bacterial meningitis); IM: inflammatory markers; LP: lumbar puncture; MRI: magnetic resonance imaging; RADT: rapid antigen detection test; RSV: respiratory syncytial virus; RT-PCR: real-time polymerase chain reaction; SARS-CoV-2: severe acute respiratory syndrome coronavirus-2; SBI: serious bacterial infection; URI: upper respiratory tract infection; UTI: urinary tract infection.*

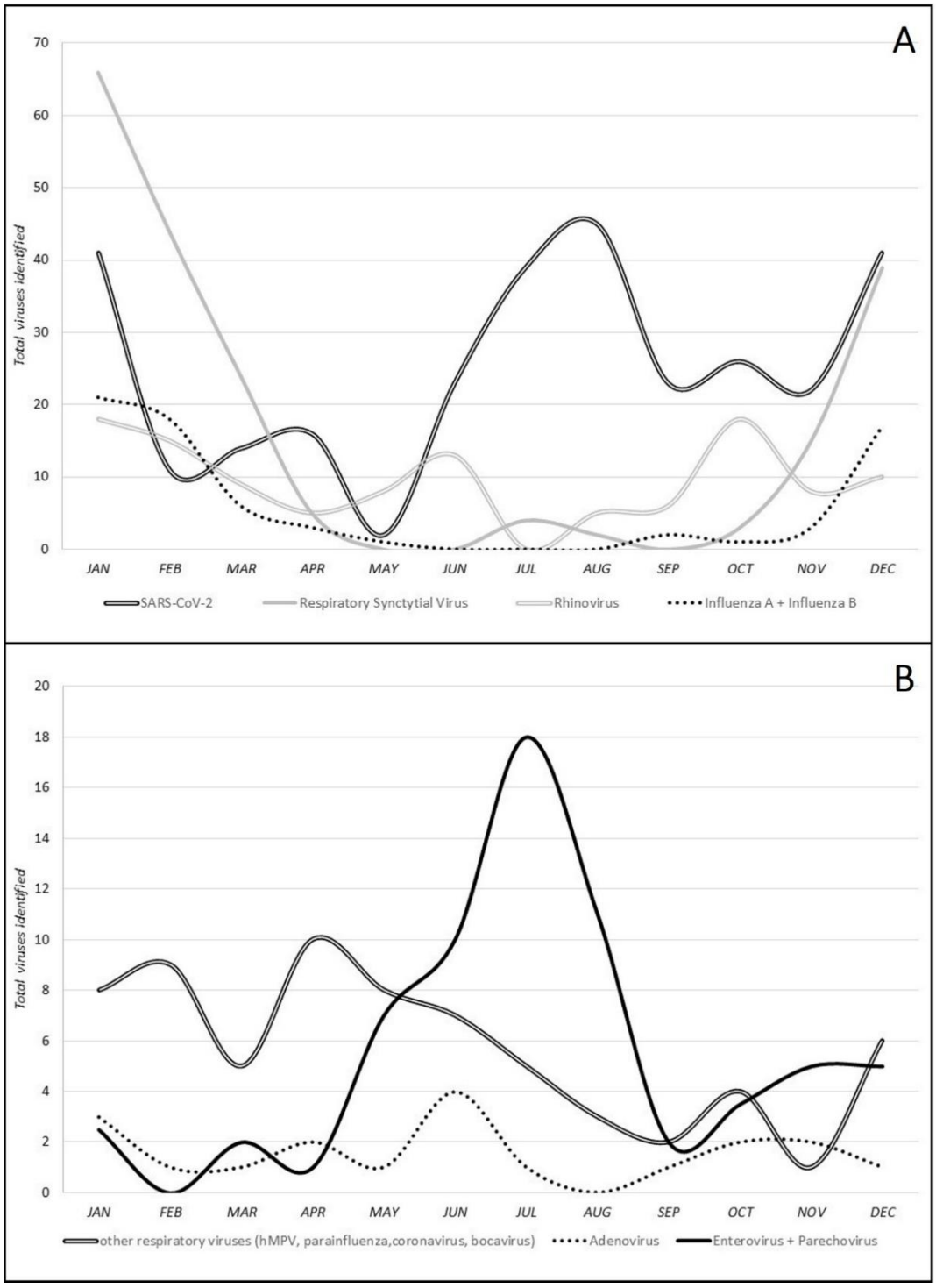

Figure 8. Seasonality of the main viruses. A. Monthly distribution of the most frequent respiratory viruses identified in febrile infants aged <90 days. **B.** Monthly distribution of less-frequent viruses identified in febrile infants <90 days of age. *hMPV; human metapneumovirus; SARS-CoV-2: severe acute respiratory syndrome coronavirus-2.* Adapted from Reference[27].

5.5.1 "major" Respiratory Viruses

Howerall, bacteremia and UTI are less frequent in young infants with respiratory viral infections than in those with negative respiratory viral tests[3,9,41,43,103-104].

Our SARS-CoV-2 cohort showed that COVID-19 in infants up to 90 days is mild, with low rates of bacterial infection, no blood-culture positivity, and excellent short-term outcomes[50,143-147]. These findings align with literature showing SARS-CoV-2-positive febrile infants, especially those older than 28 days with normal IMs, have a lower prevalence of UTI, bacteremia, and BM than virus-negative infants[50,103-104,145,148]. Thus, this subgroup of patients may be managed conservatively[50,103,143,147-148].

Influenza occupies a different position in this regard. The literature confirms a substantial disease burden of influenza in infants under 6 months, with high hospitalization rates and significant healthcare utilization, especially during seasonal peaks[3,44,49,149]. However, hospital stays are short and outcomes are generally good[44,49,150]. Screening protocols have been proposed for febrile infants aged 1–3 months. However, specific guidelines for neonates have yet to be established.[151]. Concurrently, febrile infants and neonates who test positive for influenza exhibit lower incidences of concomitant SBIs compared to those who test negative for influenza, although UTIs may still occur.[44,49,150-151]. Conversely, the prevalence of IBIs in febrile neonates with influenza infection is notably low, with no cases of BM detected.[151]. This finding is also applicable to infants aged 29–90 days, as further evidenced in our influenza cohort[49,152]. Thus, influenza serves as an excellent example of a viral diagnosis that is both clinically meaningful and risk-modifying[44,149,151]. The inclusion of rapid influenza testing for febrile young infants without focal infection signs during influenza season reduces the need for additional studies, length of stay in the PED, antibiotic use, and unnecessary hospitalizations[3,41,49,152].

While febrile infants with SARS-CoV-2 or influenza infections have a reduced risk of SBI compared to virus-negative infants, the risk of concomitant bacterial infection in febrile infants positive for human rhinovirus (hRV) by RT-PCR remains unclear. As hRV is very common in febrile infants, its detection did not reduce the risk of concomitant UTI at any age or the risk of IBI in infants aged 1–28 days. Thus, HRV detection may be relevant only in considering the risk of IBI in infants 29–90 days of age[102].

5.5.2 "minor" Respiratory Viruses

Adenovirus causes 5–10% of febrile illnesses in infants and young children and, while typically self-limiting in immunocompetent hosts, can cause severe disseminated disease in neonates, including hepatitis, pneumonia, and encephalitis, with mortality rates over 85% in disseminated forms[27,153-154]. Data on the risk of SBI co-infection in adenovirus-positive febrile infants ≤90 days of age are limited. Without specific evidence, the standard bacterial workup should not be changed based on adenovirus positivity alone. This represents a critical gap in the existing literature.

Human parainfluenza viruses (hPIVs), especially hPIV-3, cause bronchiolitis and lower respiratory tract infections in infants ≤3 months, with a generally milder course than respiratory syncytial virus (RSV)[3,9,27]. No studies have evaluated the risk of SBI co-infection in hPIV-positive febrile infants ≤90 days of age. Evidence from broader respiratory virus cohorts suggests viral positivity is linked to lower SBI risk compared to fever-only presentations, though this ca not be specifically applied to hPIV[24,27,43].

Human metapneumovirus (hMPV) causes acute respiratory illness in infants, with a similar clinical and epidemiological profile to RSV, including winter–spring seasonality and a predilection for the youngest age groups3,9,27. Although specific data on SBI co-infection risk in hMPV-positive febrile infants ≤90 days are limited, evidence from broader respiratory virus cohorts shows respiratory viral positivity is linked to a lower SBI risk compared to virus-negative cases, supporting a cautious but potentially less aggressive diagnostic approach in well-appearing infants[9,27-28,43,95].

5.5.3 Enterovirus and human Parechovirus

EV and hPeV challenge the idea that viral diagnoses are reassuring[37]. In our cohort of patients with EV/HPeV, the clinical picture often overlapped with bacterial sepsis, and serious complications were common[37-39]. Pathological MRI findings were observed in both groups, with EV and HPeV showing distinct tendencies, including higher ferritin levels in HPeV and significant inflammatory findings in EV[38]. This is entirely consistent with the literature showing that HPeV-3 in particular is strongly associated with sepsis-like illness, absent CSF pleocytosis, rash, hyperferritinemia, neurologic injury, and occasionally HLH-like or shock-like phenotypes[37-39,42,46,98,101,105-106]. Routine EV/hPeV RT-PCR testing of CSF samples in children has impacted the care of hospitalized neonates and young infants[45]. Rapid diagnosis can reduce antibiotic use and length of stay and improve patient outcomes. Thus, testing for HPeV and EV by RT-PCR on CSF or blood is indicated in febrile infants, especially neonates, without confirmed infection source and sepsis-like illnesses or suspected CNS

involvement[38-39,98,101,105-108,155-156]. This complexity is why virology must be integrated more thoughtfully into the diagnostic framework.

5.5.4 Herpes Simplex Virus

Herpes simplex virus (HSV) is unique among neonatal viral infections. It is a rare but potentially devastating disease, with a global incidence of 5–10 cases per 100,000 live births157-158. Unlike respiratory viruses, its significance is not in bacterial co-infection risk, which is low (~3%), but as an independent, urgent diagnosis requiring empirical antiviral treatment159. Neonatal HSV disease appears in three forms: skin-eye-mouth (41%), CNS encephalitis (35%), and disseminated (24%), with a mortality rate of 30% with high-dose acyclovir and up to 85% without[160-161]. Despite its rarity, the 85% mortality rate in untreated disseminated disease justifies a high suspicion in any febrile neonate ≤21 days[160-161]. Empirical intravenous acyclovir should be started immediately in febrile neonates ≤21 days with seizures, CSF pleocytosis, vesicular lesions, or unexplained elevated transaminases, pending RT-PCR confirmation[161].

5.6 Host-response biology

The unmet need in febrile infant management is not another generic IM, but a biomarker strategy capturing pathogen-specific host response[82,84,117,123-133]. The INF signature study provided strong biological evidence supporting this idea[67,90].

In our study, the IFN signature was significantly higher in non-BI than in BI and remained discriminatory even in early fever when classical IMs were weaker[67]. The AUC of the IFN score alone was high, and combining IFN with CRP or PCT yielded near-perfect discrimination in this preliminary cohort study. These findings are relevant because they suggest the host immune response contains etiologic information not captured by conventional inflammatory burden alone[15,68,84,90,117-118,130].

CRP and PCT indicate inflammation levels. Host response signatures may suggest which biological pathways are active. This is a fundamentally different and potentially more useful type of information[68]. Transcriptional biomarkers could predict and diagnose infection across viral causes and stages of disease, proving useful for guiding early therapy, quarantine, and other interventions in endemic and pandemic infectious diseases[68,124,130]. The literature on pediatric host-response biomarkers supports this direction, emphasizing the need for pathogen-discriminating tools and point-of-care strategies capable of distinguishing bacterial from viral infection rapidly and reliably[82-84,116,121-122]. Our interferon data fit within this framework and extend it to febrile infants ≤90 days, which is one of the most diagnostically difficult populations[67,120]. Simultaneously, the IFN study

highlighted the translational limitations of transcriptomic approaches. Their cost, technical complexity, processing time, and need for specialized infrastructure limit bedside utility in routine emergency workflows[84,117,119]. This gap between biological excellence and clinical practicality has motivated a shift toward CD64/CD169.

5.7 CD64 and CD169 as promising translational biomarkers

As CD169 induction on monocytes and CD64 on neutrophils is driven by type I (α) and type II (γ) IFNs, these markers should be seen as INF signature surrogates and useful in assessing viral versus bacterial immune response.[116,129-131].

To our knowledge, this is the first study to analyze both markers in a population of patients ≤90 days old. Only one study analyzed CD64 expression in 28 infants under 3 months of age, providing initial but incomplete evidence of its power in differentiating bacterial and viral infections[125]. Moreover, studies on pediatric populations in emergency departments are scarce[129,131].

Our preliminary data show a coherent pattern. Bacterial-type cases displayed a CD64-dominant profile, whereas non-bacterial/viral-type cases displayed a CD169-dominant profile, according to previous studies[126-127,130-131]. The derived ratios, particularly CD64/CD169 and its inverse, showed a strong separation between the principal groups. ROC analysis suggested excellent discriminative performance when these markers were used alone or with PCT, consistent with our previous results regarding the INF signature[67]. Thus, these leukocyte activation markers significantly distinguish bacterial from viral infections, alone or with standard, conventional IMs[129]. In addition, we analyzed the expression of CD64 and CD169 in the first infant control population, consistent with previous pediatric reports[125,162].

Figure 9 presents radar chart representations of the normalized biomarker activation profiles across study groups: panel A includes CD64, CD169, CRP, PCT, and the CD64/CD169 ratio in the BI and non-BI groups, while panel B illustrates the three-variable host-response profile (CD64, CD169, and combined ratio) across all three groups, including healthy controls.

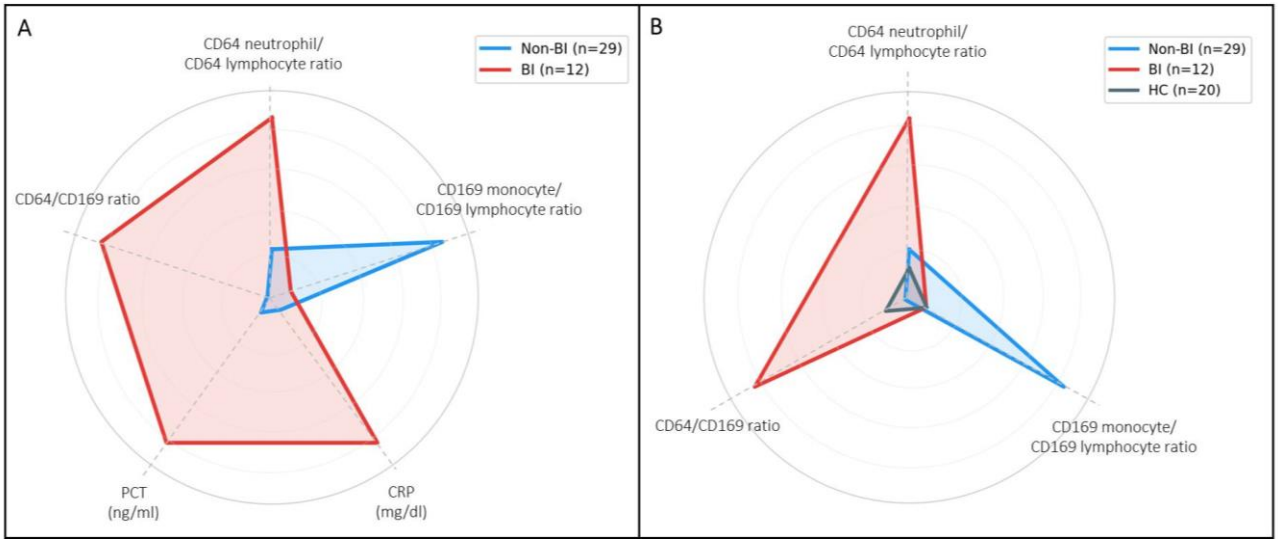

Figure 9. Radar chart representations of normalized biomarker activation profiles in febrile infants. Each axis represents a single biomarker, with values normalized to the maximum observed median across the compared groups for each variable (100% = highest group median), enabling a direct visual comparison of relative biomarker activation, irrespective of absolute scale differences. Colored lines connect the normalized median values for each group, forming the corresponding diagnostic signature polygon. The shaded areas represent the magnitude of the overall activation profile. **Panel A:** Five-variable profile including CD64 ratio (neutrophil CD64/lymphocyte CD64), CD169 ratio (monocyte CD169/lymphocyte CD169), C-reactive protein (CRP, mg/dL), procalcitonin (PCT, ng/mL), and the combined CD64/CD169 ratio, comparing the non-bacterial infection (non-BI, blue, n=27) and bacterial infection (BI, red, n=12) groups. The BI group displayed a broadly elevated profile with marked predominance of CD64, CRP, PCT, and CD64/CD169 ratio, consistent with a bacterial inflammatory phenotype. The non-BI group showed predominant CD169 expression with uniformly low values across all remaining axes, reflecting interferon-mediated monocyte activation in the absence of significant bacterial inflammatory stimulation. **Panel B:** Three-variable host-response profile (CD64, CD169, and CD64/CD169 ratio) across all three study groups, including HC. The HC group was characterized by a uniformly low baseline expression across all markers. Healthy controls were excluded from the left panel as CRP and PCT values were not available for this group. Values are normalized per variable; percentage rings indicate 25%, 50%, 75%, and 100% of the maximum observed median. *BI: bacterial infection group; CRP: C-reactive protein; HC: healthy control group; non-BI: non-bacterial infection group; PCT: procalcitonin.*

CD169 is linked to interferon-mediated activation, while CD64 reflects myeloid activation typical of bacterial inflammation or disease[126-127,131]. Consistent with previous pediatric results, our findings suggest that CD64 and CD169 could become novel biomarkers to guide the early diagnosis and treatment of febrile infants[129,131]. Moreover, the simultaneous assessment of both markers could offer a real-time evaluation of the patient's immune status and risk of superimposed bacterial infections[132]. The identification of data-driven cutoffs offers a clinically meaningful framework. While predefined analytical thresholds (CD64 ratio >8 and CD169 ratio >4) are useful for laboratory standardization, our ROC-derived thresholds better reflect the diagnostic performance in

these populations. The superior discriminatory performance of the CD64/CD169 ratio (AUC 0.989) over either IM alone supports the measurement of both markers in clinical practice rather than relying on a single host-response biomarker. CD64 and CD169 are regulated by distinct interferon pathways, IFN-γ and IFN-α, respectively. Their combination captures both the bacterial activation signal and viral response suppression within a single metric, amplifying the discriminatory signal beyond what either pathway can provide alone[129-131].

It has also been suggested that their combined use enables reliable bacterial-viral differentiation in immunocompromised patients and neonates, where traditional IMs lack specificity[129-130].

Table 5 compares the diagnostic characteristics and performance of conventional IMs and novel host-response markers evaluated in this study, highlighting the biological basis, activation kinetics, guideline-recommended and ROC-derived cut-offs, and discriminatory performance across bacterial and non-bacterial infection groups.

Biomarker	Type	Trigger	Timing of Peak	AUC	Optimal Cut-off	Sensitivity / Specificity (%)	Advantages and Limitations
WBC [12,23,30,52,55-57, 63,69-70,81,85-86]	Conventional blood cell count	Non-specific inflammation For both bacterial and viral infections	Rapidly elevated in h Nnon-specific kinetics	~0.60–0.65	5,000–15,000 ×10³/L *Rochester, NICE*	40–70% / 40–60%	Universally available Inexpensive and rapid Integrated in historical algorithms Affected by age norms, stress, immunisation Low sensitivity for early infection
ANC [12,23,30,52,63,69-70,85-86]		Bacterial infection (primary) Myeloid activation	Rapidly elevated in h Slightly more specific than WBC but similar kinetics	~0.65–0.72	≤4,090/mm³ *PECARN* ≤4,000–5,200/mm³ *AAP*	60–80% / 50–65%	Better specificity than WBC Integrated in PECARN and AAP Associated with improved IBI detection Lower sensitivity in bacteraemia and GBS
CRP [12,20,22-23,26,30-31, 52,63,69-72,88,91]	Conventional acute-phase protein	IL-6–mediated hepatic synthesis Bacterial > viral infections	Peak at 24–36 h from fever onset Falsely low in first 6–12 h	~0.70–0.89 (SBI)	<20 mg/L *SBS, AAP* ≤75 mg/L *(PCT adjustment) Lab Score*	70–85% / 55–75%	Widely available and well-validated Cost-effective Good NPV when combined with PCT Falsely low in first 6–12 h Non-specific in distinguishing bacterial vs viral
PCT [12,20,22-23,26,30-31,52,62-63,67,69-70,85-86,88,91,130]		Bacterial endotoxin and pro-inflammatory cytokines Partially suppressed by IFN-α in viral infections	Peak at 8–24 h from fever onset More rapid than CRP	~0.85–0.94 (IBI)	<0.5 ng/mL *SBS, AAP* ≤1.71 ng/mL *PECARN*	75–90% / 65–80%	Best conventional biomarker for IBI Incorporated in all major modern algorithms Associated with improved risk stratification Falsely low in first 6–8 h Less sensitive for UTI than for IBI Not universally available and higher cost
CD64 Ratio [67,125,127-131,163]	Host-response flow cytometry	IFN-γ–mediated upregulation in bacterial infection	Elevated within 6 h of bacterial stimulus Precedes CRP and PCT peaks	0.922 *this study*	predefined cut-off >8 ≥12.63 *ROC-derived, Youden index, this study*	~83–92% / ~88–95% ~100%/~69% *this study*	Rapid bacterial-specific signal Elevated earlier than CRP/PCT Routine EDTA tube without additional blood sampling Less affected by non-infectious inflammation Flow cytometry required Limited validation in febrile infants Preliminary data only
CD169 Ratio [67,123,126,129-131,133,163]		IFN-α–mediated upregulation in viral infection	Elevated within 8 h of viral stimulus Independent of CD64 pathway	~0.91 (inverse) *this study*	predefined cut-off >4 *for viral positivity* ≤12.78 *ROC-derived, inverse direction, this study*	~80–90% / ~85–92% ~92%/~93% *this study*	Specific viral-response marker Provides etiologic info complementary to CD64 Inverse-direction interpretation required for BI Flow cytometry required Preliminary data in febrile infants
CD64/CD169 Ratio [67,129-131,133,163]		Integrates both bacterial (CD64) and viral (CD169) signals *Ratio amplifies discrimination between infection types*	Reflects combined IFN-γ and IFN-α kinetics Both markers activated independently	0.993 *this study*	≥1.27 *ROC-derived, Youden index, this study*	~100%/~96.6% *this study*	Superior discriminatory performance Etiologic rather than severity-based Additional gain with CRP/PCT Requires simultaneous CD64 and CD169 External validation in febrile infants pending Interpretability requires specialist training

Table 5. Characteristics and diagnostic performance of inflammatory biomarkers in febrile infants ≤90 days of age. Superscript numbers refer to the bibliography as numbered in the thesis. The CD64 ratio refers to the neutrophil CD64/lymphocyte CD64 ratio, and the CD169 ratio refers to the monocyte CD169/lymphocyte CD169 ratio. The AUC values for WBC, ANC, CRP, and PCT represent the ranges reported in external validation cohorts of febrile infants ≤90 days. The AUC, sensitivity, and specificity values for the CD64, CD169, and CD64/CD169 ratios were derived from the present study; external validation is pending. Sensitivity and specificity estimates for conventional biomarkers are approximate ranges across published studies using varying cut-offs and populations. For CD169, inverse-direction ROC analysis was applied, and lower CD169 ratio values indicated bacterial infection. CD64/CD169 optimal cut-off (≥1.27) derived from Youden index maximization. The sensitivity/specificity column reports the ranges. The CD64/CD169 ratio showed the highest AUC among all the biomarkers tested. *AAP: American Academy of Pediatrics; ANC: absolute neutrophil count; AUC: area under the ROC curve; BI: bacterial infection; CRP: C-reactive protein; EDTA: Ethylene-diamine-tetraacetic acid (*standard blood collection tube*); GBS: Group B Streptococcus; HC: healthy control; IBI: invasive bacterial infection; IFN: interferon; NICE: National Institute for Health and Care Excellence; NPV: negative predictive value; PCT: procalcitonin; PECARN: Pediatric Emergency Care Applied Research Network; ROC: receiver operating characteristic; SBI: serious bacterial infection;SBS: Step-by-Step algorithm; UTI: urinary tract infection; WBC: white blood cell.*

Incorporating conventional IMs into multivariate logistic regression models with CD64 and CD169 further increased the AUC values. The combination of CD64, CD169, and PCT achieved an AUC of 0.997, whereas the four-marker model with CRP reached a nominally perfect AUC of 1.000 (**Figure 7B**). These results should be interpreted with considerable caution, as the small sample size of the infectious subgroup (n=41) renders these combined models highly prone to overfitting, and their performance in independent cohorts remains uncertain. Nevertheless, the directional finding is biologically plausible and suggests that integrating host response markers with conventional acute-phase IMs may provide incremental diagnostic value. Thus, this hypothesis warrants prospective evaluation in adequately powered, multicentric studies. Efforts to discover, validate, and implement such biomarkers align with global antimicrobial stewardship goals and represent a step towards precision pediatrics, where objective molecular evidence of host/pathogen interactions should play a leading role[90].

6. CLINICAL IMPLICATIONS

The clinical implications of this doctoral research are substantial.

First, the data suggest that febrile infants should not be managed using a static model. Time from fever onset should be explicitly incorporated into biomarker interpretation and clinical reasoning[26,31,67-69].

Second, viral testing should be performed more strategically. Evidence from this thesis and the supporting literature suggests viral diagnostics can reduce unnecessary testing, antibiotic exposure, and hospitalization burden, especially when integrated into structured care pathways[2,3,10,35,41,62,98].

Third, certain viruses require targeted clinical awareness. HPeV and EV should be considered in young infants with sepsis-like illness, abnormal neurologic features, rash, or hyperferritinemia, especially in seasonal contexts[28,38-39,41,44,101-102,106-107]. Similarly, influenza and SARS-CoV-2 have distinct implications for bacterial co-infection risk and management intensity[9,24,50,94-95,103,143,148].

Fourth, host-response biomarkers may be the most promising method for reducing diagnostic uncertainty without compromising safety. If validated, CD64 and CD169 ratios could help identify infants more likely to have bacterial versus viral biology, particularly when conventional IMs are equivocal or obtained early*90,125-127,129-131*. Furthermore, its point-of-care application with minimal blood could represent a turning point in the management of febrile infants[132,163].

Table 6 summarizes the main findings.

Clinical Area	Key Points	Clinical Implications
Dynamic interpretation of febrile infants	Biomarkers and clinical assessment should not be static Time from fever onset significantly affects interpretation	Clinicians should integrate timing of fever into decision-making, not rely only on single lab values
Strategic viral testing	Viral diagnostics can reduce unnecessary blood tests, antibiotic use, and hospital admissions when used within structured pathways	Encourage targeted, protocol-driven viral testing to improve resource use and reduce overtreatment
Virus-specific clinical awareness	Some viruses (HPeV, EV) are strongly linked to sepsis-like illness, neurologic symptoms, rash, or hyperferritinemia Influenza and SARS-CoV-2 have distinct bacterial co-infection risks	Maintain high suspicion for specific viruses in characteristic clinical presentations and seasonal contexts
Host-response biomarkers (CD64/CD169)	Promising tools to differentiate bacterial and viral infections, especially when traditional markers are unclear or in the early stages of the disease. Potential point-of-care use with small blood volume	Could significantly reduce diagnostic uncertainty and improve early triage decisions in febrile infants

Table 6. Summary of the main translational insights across four interconnected clinical domains. The first domain, dynamic interpretation of febrile infants, emphasizes that both biomarkers and clinical assessment must be interpreted in relation to the timing of fever onset, as single laboratory values obtained early in the febrile course may significantly

underestimate the inflammatory response and should not be used in isolation to guide clinical decision-making. The second domain, strategic viral testing, highlights that targeted, protocol-driven viral diagnostics, when integrated into clinical pathways, may reduce unnecessary laboratory investigations, antibiotic prescriptions, and hospital admissions without increasing the rate of missed bacterial infections. The third domain, virus-specific clinical awareness, underscores the importance of maintaining heightened clinical suspicion for specific viral pathogens associated with severe or atypical presentations, which can manifest as sepsis-like illness, neurological involvement, or haemophagocytic lymphohistiocytosis in the absence of the usual inflammatory biomarker elevation. The fourth domain, host-response biomarkers, presents the translational potential of these markers as complementary tools for differentiating bacterial from viral infections at the bedside. Their biological specificity, early elevation kinetics, and superior discriminatory performance compared to conventional inflammatory markers suggest that their integration into clinical decision-making frameworks could reduce diagnostic uncertainty and improve early triage in febrile infants ≤90 days of age, pending external validation in independent cohorts. *EV: enterovirus; HPeV: human parechovirus; SARS-CoV-2: severe acute respiratory syndrome coronavirus-2.*

6.1 Strengths

This study has several strengths. It is not based on a single study but on a coherent research program integrating epidemiology, clinical virology, IMs kinetics, transcriptomics, and membrane biomarker analysis. Another strength of this study is the continuity of the setting. The studies were mostly conducted within the same tertiary pediatric emergency environment, supporting internal coherence and enabling the progressive refinement of research questions. A third strength is the translational trajectory. This research progresses from describing the clinical problem to biological proof-of-concept to a potentially applicable bedside biomarker strategy.

6.2 Limitations

This study had several limitations that must be acknowledged. Many components are single-center, reflecting local epidemiology, testing strategies, and institutional management behavior. The retrospective design introduces the risk of incomplete testing, missing data, and selection bias. Viral testing was not systematic throughout all years, possibly underestimating some viral diagnoses in the earlier phases. Clinically presumed viral groups, which are necessary for real-world retrospective analyses, exhibit some diagnostic heterogeneity. The interferon signature study, despite its importance, included a small number of patients, especially in the bacterial group. The CD64/CD169 study was preliminary and exploratory, and its excellent diagnostic performance must be interpreted cautiously until replicated in larger, independent populations. Although the sample size was limited, the proportion of bacterial infections in our cohort was higher than that typically reported, possibly influencing the diagnostic performance and overestimating the discriminative ability of the evaluated biomarkers. Additionally, analysis was not possible 24/7, depending on laboratory availability. Moreover, the median time from fever onset to sampling exceeded 12

hours, potentially limiting its applicability to early presentations. Finally, conventional IMs were unavailable in the control group, which limited comparisons across all groups.

6.3 Future directions

Future studies should evaluate CD64 and CD169 in larger populations, different epidemiological settings, and clinically relevant subgroups, such as neonates versus older infants, early versus late presenters, viral-test-positive versus viral-test-negative infants, and mixed bacterial-viral phenotypes. These studies should assess these markers not in isolation but as part of integrated diagnostic models, including timing, age, urinalysis, and epidemiological context.

Equally important, future research should examine whether these biomarkers can improve clinically important outcomes, such as reducing unnecessary lumbar punctures, safer antibiotic de-escalation, shorter LOSs, and better recognition of severe viral phenotypes.

In parallel, a point-of-care platform is currently under development for rapid CD64 and CD169 assessment using micro-sample real-time FC techniques in the PED, which can overcome conventional laboratory limitations and reduce turnaround times[132,163]. This method could complement CPR and PCT point-of-care micromethods with a minimally invasive, needle-free approach and minimal blood sampling.

The goal should be to build a clinically safe and usable model for febrile infant assessment that is both biologically grounded and operationally realistic.

7. CONCLUSIONS

This doctoral research supports three main conclusions.

First, the current bacteria-centered approach to febrile infants is safe but lacks specificity.

Second, viral infections should be central to the febrile infant paradigm. They are the predominant etiologies, clinically heterogeneous, and influence bacterial risk without completely abolishing it.

Third, host response biomarkers offer a promising path for a more accurate diagnostic framework. The interferon-signature study showed the biological validity of this approach, while the preliminary combined assessment of CD64 and CD169 is a biologically grounded, clinically promising method for early discrimination of bacterial and non-bacterial infections in febrile infants.

The shortfall of today's diagnostic capabilities results in the risk of morbidity, mortality, antibiotic overuse, and avoidable healthcare costs. Novel biomarkers, such as IFN scores and CD64 and CD169 ratios, could reduce this burden in young infants and families. Our results suggest a shift from a static, bacteria-centered paradigm to an integrated model based on timing, viral epidemiology, and host response biology. If confirmed in larger studies, this strategy could improve risk stratification and support targeted management, reducing unnecessary antibiotic use and hospitalizations, and better reflecting the complexity of fever in the first 90 days of life.

8. ABBREVIATIONS

AAP	American Academy of Pediatrics
ANC	Absolute Neutrophil Count
AUC	Area Under the Curve
BI	Bacterial Infection group
BM	Bacterial Meningitis
CBE	Clinically Benign Episode
CFU	Colony-forming Unit
CNS	Central Nervous System
CRP	C-Reactive Protein
CSF	Cerebrospinal Fluid
EDTA	Ethylene-diamine-tetra-acetic acid
EV	Enterovirus
FC	Flow Cytometry
FSC-A	Forward Scatter Area
FWS	Fever without Source
GBS	Group B Streptococcus
GeoMFI	Geometric Mean Fluorescence Intensity
HC	Healthy Control
Hib	Haemophilus influenzae type B
hPeV	human Parechovirus
hMPV	human Metapneumovirus
hPIV	human Parainfluenza Virus
hRV	human Rhinovirus
HSV	Herpes Simplex Virus
IBI	Invasive Bacterial Infection
IM	Inflammatory Biomarker
INF	Interferon
IQR	Interquartile Range
LP	Lumbar Puncture
MRI	Magnetic Resonance Imaging
NICE	National Institute for Health and Care Excellence

Non-BI	non Bacterial Infection group
PCT	Procalcitonin
PED	Pediatric Emergency Department
RT-PCR	Real Time Polymerase Chain Reaction
ROC	Receiver Operating Characteristic
PECARN	Pediatric Emergency Care Applied Research Network
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus-2
SBI	Serious Bacterial Infection
SBS	Step-by-Step
SSC-A	Side Scatter Area
UTI	Urinary Tract Infection
WBC	White Blood Cell

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10. APPENDIX

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ACTA PÆDIATRICA NURTURING THE CHILD WILEY

BRIEF REPORT

Repeated inflammatory markers may be useful for assessing febrile infants aged 29–90 days during early hospital surveillance

Fever is a frequent reason for paediatric emergency room (PED) visits. Most are viral infections, but up to 12% of children aged up to 90 days have a serious bacterial infection (SBI).[1,2] Physical examinations and laboratory tests do not always differentiate between these. The algorithms developed to identify subgroups at higher risks of SBIs in this age group include the validated Step-by-Step, which has identified a lower incidence of undiagnosed SBIs than others.[1–3] This approach evaluates the risk of SBIs in febrile infants by their general appearance, age, urinalysis, procalcitonin, C-reactive protein (CRP) and absolute neutrophils counts (ANC). However, it appears to be less sensitive in children with short fevers.[2,3] This retrospective cohort study explored how reliably early blood biomarkers detected SBIs in febrile infants.

We assessed the medical records of infants aged 29–90 days with a fever of ≥38°C who presented to our PED from 1 January 2018 to 31 December 2020. We enrolled patients with fever measured at home, but excluded afebrile patients whose suspected fever was only measured on arrival by touch.[4,5] The Pediatric Assessment Triage identified children who seemed well using appearance, respiratory work and circulation.[4] The pathological Step-by-Step values were: CRP >20 mg/L, procalcitonin >0.5 ng/mL and ANC >10 000 cell/mm^3.[3] Patients were divided into two groups. First, infants with SBIs detected by positive cultures in any usually sterile sites: bacteraemia, meningitis, urinary tract infections, bone infections, bacterial pneumonia and skin/soft tissue. Second, infants with clinical benign episodes, namely microbiologically-confirmed viral infections or culture-negative suspected viral infections. Patients with SBIs were divided into ≤12 h of fever and >12 h. Patients with incomplete blood tests or cultures were excluded.

Group differences were measured by the Kruskal–Wallis or Mann–Whitney *U*-test for continuous variables and the chi-square or Fisher's exact test for categorical variables. A two-tailed *p*-value of <0.05 was statistically significant. The statistical analysis used SPSS 20 for Windows, (SPSS Inc.).

We recruited 456 febrile patients aged 29–90 days, but excluded 148 due to incomplete medical data, incomplete follow-up or unknown final diagnosis. The remaining 308 children (66.5% male) comprised 90 (29.2%) with SBIs and 218 with clinical benign episodes. The SBI patients were divided into 56 (62.2%) with fever ≤12 h and 34 (37.8%) with fever >12 h. Table 1 compares the inflammatory markers values among the three groups. We found differences in ANC, CRP and procalcitonin values between the SBI ≤ 12 h and SBI > 12 h groups ($p = 0.006$, $p = 0.0001$, $p = 0.0001$ respectively). We also found a statistically significant difference in all biomarkers between the CBE group and the SBI > 12 h group and a statistically significant difference for ANC between the CBE and SBI ≤ 12 h group.

Our data suggest that performing inflammatory markers at <12 h in feverish children aged 29–90 days did not exclude SBIs, and may be misinterpreted, but performing them after 12 h from fever onset reliably identified SBI events. No differences were observed between early and late markers for clinical benign episodes, reinforcing our conclusion.

Children aged ≤90 days are often brought to the PED very quickly[2] and it may be difficult to differentiate between clinical benign episodes and SBIs with just inflammatory markers. CRP and procalcitonin are the most commonly collected inflammatory markers in blood, with peak levels at 24–36 and 8–24 h of fever respectively. They should be used carefully to stratify infectious risks, especially in children with short fevers.[2]

Although it did not reach statistical significance ($p = 0.06$), the absolute value of procalcitonin between the SBI < 12 h and clinical benign episode group was borderline significant. This suggests that procalcitonin may be the earliest, most reliable biomarker in the first hours of fever, even if an hourly cut-off has not yet been identified.

A debatable point may be the age limit of the enrolled patients. The American Association of Pediatrics guidelines on managing febrile infants aged ≤60 days[6] state that children under 28 days reportedly face twice the risk of SBIs than older children. That is why we excluded neonates from our study. However, the prevalence of SBIs in febrile infants aged 61–90 days was not significantly lower than in infants aged <60 days. Therefore, we believe that all children from 29 to 90 days of age can be considered as one group.[4,6]

Our retrospective study had the usual limitations of this method and the high number excluded due to incomplete data could have led to a selection bias.

Abbreviations: ANC, absolute neutrophil count.; CRP, C-reactive protein; PED, paediatric emergency room; SBI, serious bacterial infection.

TABLE 1 Significant differences in inflammatory marker values among the three groups.

	All	SBI ≤12 h fever (group no. 1)	SBI > 12 h fever (group no. 2)	CBE (group no. 3)	1 vs. 2	1 vs. 3	2 vs. 3
	N = 308	*N* = 56	*N* = 34	*N* = 218	*p* Value	*p* Value	*p* Value
Gender, Male	205 (66.6)	39 (69.6)	27 (79.4)	139 (63.8)	0.34	0.44	0.08
ANC, absolute values	5081.16 ± 3884.21	6336.27 ± 4044.14	9318.59 ± 6058.07	4097.86 ± 2725.22	**0.006**	**0.0001**	**0.0001**
ANC, >10 000 cell/mm^3	34 (11.0)	10 (17.9)	13 (38.2)	11 (5.0)	**0.05**	**0.003**	**0.0001**
CRP, absolute values	3.01 ± 4.05	2.25 ± 2.46	9.49 ± 6.89	2.20 ± 2.64	**0.0001**	0.63	**0.0001**
CRP, >20 mg/L	138 (45.0)	23 (41.1)	29 (85.3)	86 (39.6)	**0.0001**	0.88	**0.0001**
PCT, absolute values	2.00 ± 5.62	1.77 ± 3.98	7.78 ± 12.06	0.76 ± 1.97	**0.0001**	0.06	**0.0001**
PCT, >0.5 ng/mL	87 (41.6)	19 (35.2)	24 (82.8)	44 (34.9)	**0.0001**	1	**0.0001**

Note: Mean ± SD; *N* (%). Data are described as means and standard deviations for continuous variables and numbers and percentages for categorical variables. Bold indicates significant *p*-values.

Abbreviations: ANC, absolute neutrophils count; CBE, clinically benign episode; CRP, C-reactive protein; PCT, procalcitonin; SBI, severe bacterial infection.

The prevalence of SBIs was high enough to recommend systematic blood tests in children aged 29–90 days admitted to PEDs, but inflammatory markers before 12 h of fever may not differentiate clinical benign episodes and SBI. The latter may then be under-recognised. Paediatricians should be aware of this risk. We recommend hospital surveillance for at least 12–24 h with repeated inflammatory markers.[1] Larger, prospective studies need to assess whether a time variable would improve the efficacy of the Step-by-Step approach and improve the management of febrile infant in PEDs.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

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Seasonal and Age-Specific Patterns of Viral and Bacterial Infections in Febrile Infants ≤90 Days Presenting to a Paediatric Emergency Department

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ABSTRACT

Aim: Febrile infants ≤90 days are at increased risk of serious and invasive bacterial infections (SBIs/IBIs), although viral illnesses account for most febrile episodes. Current management algorithms prioritize bacterial risk stratification, while viral epidemiology is inconsistently integrated into pathways. Aim of this study is to describe age-specific bacterial and viral etiologies, seasonality, temporal trends, and co-infections in febrile infants presenting to a tertiary paediatric emergency department. **Methods:** retrospective observational study including infants ≤90 days evaluated between 2018-2025. Infants were stratified into three age groups (≤28, 29–60, 61–90 days). Bacterial and viral etiologies, co-infections, seasonal distribution, and temporal trends were analyzed. **Results:** Among 1,529 infants, SBI represented 15% of cases. IBI were age-dependent, peaking in neonates ≤28 days, whereas viral infections predominated across all groups. Approximately 10% of SBIs occurred with documented viral co-infection. Bacterial infections showed no seasonal pattern, while viral infections demonstrated seasonality, with winter respiratory peaks and summer enterovirus/parechovirus circulation. Viral detections declined during 2020–2021 and rebounded in 2022 whereas bacterial rates remained stable. **Conclusions:** Viral etiologies display pronounced seasonality, whereas bacterial infections remain non-seasonal. Viral positivity does not exclude SBI and urinalysis should remain routine. An epidemiology-aware approach may support without replacing bacterial risk stratification.

KEYWORDS

Bacterial Infections; Co-infection; Fever; Infant, Newborn; Seasonality; Urinary Tract Infections; Virus Diseases

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SUMMARY

- Viral infections are the predominant cause of fever in young infants and show marked seasonal variation, whereas serious bacterial infections remain stable throughout the year.
- Invasive bacterial infections are strongly age-dependent, peaking in neonates and declining with older age.
- Viral positivity reduces but does not eliminate the risk of concurrent bacterial infection, particularly urinary tract infections, supporting an age-driven approach without replacing systematic bacterial evaluation.

ABBREVIATIONS

CFU	colony forming unit
CSF	cerebrospinal fluid
IBI	invasive bacterial infection
LBI	localized bacterial infection
PECARN	pediatric emergency care applied research network
PED	pediatric emergency department
POCT	point-of-care testing
RSV	respiratory syncytial virus
RT-PCR	real time polymerase chain reaction
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SBI	serious bacterial infection
UTI	urinary tract infection

INTRODUCTION

Febrile infants younger than 90 days are at significantly higher risk of serious and invasive bacterial infections (SBIs and IBIs) compared with older children, owing to immunologic immaturity, lack of complete vaccination protection, and potential perinatal pathogen exposure (1–6).

The risk of IBIs is highest during the first month of life and progressively decreases with age, although it does not completely disappear in older infants (4,6). Because fever may be the only presenting symptom and clinical examination alone is insufficient to reliably exclude bacterial disease, structured diagnostic pathways have been developed to guide their management (1-8). Clinical prediction rules such as Pediatric Emergency Care Applied Research Network (PECARN), Step-by-Step, and American Academy of Pediatrics criteria aim to identify infants at risk for SBIs/IBIs based on age, clinical appearance, urinalysis, and inflammatory biomarkers (1,5,8-11). Consequently, many febrile infants undergo extensive diagnostic evaluation, including lumbar puncture, empirical broad-spectrum antibiotics, and hospital admission, even when clinically well appearing (1-3,12).

Despite their widespread implementation, current algorithms primarily focus on ruling in or ruling out bacterial infections. Viral testing plays a secondary or optional role, although viral illnesses account for the majority of febrile episodes in this age group (7,9,13). Febrile infants with confirmed viral infections have a lower yet non-negligible, risk of concomitant SBI compared with virus-negative infants, but available studies are still limited (9,14). However, viral diagnostics are not systematically integrated into most decision-making pathways, and viral testing strategies often vary according to local practice, availability, and seasonal patterns (9).

As a result, distinguishing the minority of infants with SBI/IBI from the large proportion with self-limiting viral infections remains a central critical challenge, particularly given the non-specific clinical presentation and the potential for rapid clinical deterioration in early infancy (1-5,9,13-14).

Although previous studies have examined bacterial risk or specific viral infections separately, few have simultaneously described bacterial and viral etiologies across age groups, seasons, and calendar years within the same cohort. A comprehensive

epidemiological characterization integrating age stratification, seasonal variation, and temporal trends may provide clinically relevant context for risk assessment without compromising patient safety (14-15). The present study aimed to describe the distribution of bacterial and viral etiologies in febrile infants ≤90 days presenting to a tertiary pediatric emergency department (PED). We analyzed age-specific risks, seasonal patterns, temporal trends, and rates of bacterial–viral co-infection with the goal of contextualizing viral diagnostics within established bacterial risk frameworks while maintaining a conservative approach to bacterial evaluation.

MATERIALS AND METHODS

Study design and setting

We conducted a retrospective observational study at the PED of IRCCS Giannina Gaslini, a tertiary-level paediatric hospital located in Genoa, Italy, managing approximately 38,000 PED admissions annually. The study encompassed febrile infants younger than 90 days who were evaluated between January 2018 and December 2025.

We included all infants aged ≤90 days presenting to the PED with documented fever, defined as a measured axillary temperature >37.5°C or rectal temperature ≥38°C, either confirmed upon admission or objectively documented at home within 12 hours prior to presentation.

We excluded all patients with fever reported solely by touch and not confirmed in PED, infants with fever occurring within 48 hours after immunization or with a clearly identified non-infectious cause.

All infants were managed according to institutional clinical pathways consistent with international guidelines, including the Step-by- Step approach (9). Enrollment was extended to 90 days of life in line with our institutional protocol which considers comparable prevalences of IBI in febrile infants aged 29–60 and 61–90 days (4-6,8-9).

Infants were stratified into three predefined age groups: ≤28 days, 29–60 days, 61–90 days. The ≤28-day cutoff was selected as it reflects institution routine clinical practice (5,8).

SBIs are defined as the presence of bacterial meningitis or bacteremia or urinary tract infection (UTI) and IBIs as the presence of bacterial meningitis or bacteremia. Bacteremia and bacterial meningitis were defined as the growth of a single pathogen in

the blood or cerebrospinal fluid (CSF), respectively. UTI was defined as the growth of a single pathogen in the urine with colony counts (colony-forming units, CFUs) ≥100.000 CFUs/mL, in association with an abnormal dipstick test or urinalysis (positive for leukocytes, leukocyte esterase, or nitrites). Blood and CSF cultures were considered contaminated when organisms not commonly accepted as pathogens were isolated.

Localized bacterial infections (LBIs) were defined as a clear source of bacterial infection (osteomyelitis, septic arthritis, lymphadenitis, omphalitis, or pertussis) confirmed by microbiological identification. LBIs with positive blood cultures were subsequently classified as IBI.

For greater clarity, throughout the manuscript the term "IBI" refer exclusively to bacteremia and bacterial meningitis, whereas "SBI" include both IBIs and UTIs, which represent the most frequent non-invasive bacterial diagnosis in this age group.

Viral testing was performed based on clinical presentation, seasonal epidemiology, and local availability. Testing strategies evolved over the study period. Rapid antigenic respiratory viral testing included Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), Respiratory Syncytial Virus (RSV), Influenza A/B, Adenovirus. Detection methods included rapid antigen tests, point-of-care molecular assays, and Real Time Polymerase Chain Reaction (RT-PCR). Additional respiratory viruses (rhinovirus, metapneumovirus, parainfluenza, bocavirus, seasonal coronaviruses) were tested by RT-PCR when clinically indicated.

Cerebrospinal fluid RT-PCR testing for viruses and bacteria was performed in selected cases depending on age and clinical findings. The diagnostic panel included the following pathogens: *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Listeria monocytogenes*, *Escherichia coli*, *Streptococcus agalactiae*; herpes simplex virus type 1 and type 2, human herpesvirus 6 and 7, varicella-zoster virus, Epstein–Barr virus, cytomegalovirus, human parechovirus, enterovirus, adenovirus, mumps virus, and parvovirus B19.

Other viral pathogens (e.g., rotavirus, norovirus) were tested based on clinical suspicion. From 2020 onward, point-of-care testing (POCT) for SARS-CoV-2 was introduced, followed in late 2021 by POCT for influenza, RSV, and adenovirus. Broader RT-PCR panels continued to be used selectively, particularly in hospitalized infants or when POCT results were negative but further etiologic clarification was clinically indicated.

Each infant was classified into one of the following mutually exclusive etiological categories: IBI, UTI, LBI, confirmed viral infection, presumptive viral infection. Additionally, co-infections involving viral agents were also considered in the context of bacterial infections. A presumptive viral infection was defined as a clinical syndrome consistent with viral illness without microbiological confirmation, and with a normal urinalysis.

Seasonality was assessed using monthly aggregation of etiologic categories. Temporal trends were evaluated on a yearly basis from 2018 to 2025.

Statistical analysis

Categorical variables are reported as absolute frequencies and percentages, and continuous variables are presented as medians and interquartile ranges. Analyses were descriptive and aimed to characterize etiologic distributions rather than identifying indipendent predictors. To evaluate differences between groups, the Kruskal-Wallis test or Mann–Whitney U-test was employed for continuous variables, and the chi-square or Fisher's exact test was utilized for categorical variables. A two-sided p value <0.05 was considered statistically significant. All statistical analyses were performed using Graph Pad Prism version 9.1.0 for Windows (Graphpad Software, San Diego, California, USA, www.graphpad.com).

RESULTS

A total of 1,529 patients were enrolled. Fourteen patients were excluded, with 13 exclusions due to a post-immunization fever and one due to Kawasaki disease. The prevalence of UTI was consistent across age groups with an overall rate of 11%. However, IBIs demonstrated a clearly age-dependency, peaking in neonates (≤28 days: 6.9%) and declining with age (61–90 days: 1.6%) (Table 1). Only one bacterial meningitis was identified in the 29-60 days age group. Viral infections were the most common etiologies, but the 61–90-day age group showed fewer documented viral infections (46.7%) and a higher proportion presumptive viral infections (34.7%), compared to younger groups (Table 1). Co-infections were uncommon overall. UTIs with viral co-infection occurred in 1.1% of all infants, corresponding to approximately 10% of all UTIs

(Table 1). Additional sensitivity analyses using a ≤21-day cutoff revealed no significant differences between infants aged 0–21 and those aged 22–28 days.

SARS-CoV-2 detection varied by age and was highest in 29–60 days group (41.3%), while lower in infants aged ≤28-days (Table 2). Enterovirus and parechovirus revealed strong age gradients, with the highest proportions in ≤28 days group (enterovirus 14.3%; parechovirus 4.9%) (Table 2). Influenza A/B did not differ significantly across age groups (Table 2).

Throughout the months, bacterial infections (IBIs, UTIs, and LBIs) remained relatively stable, showing no clear seasonal pattern (Figure 1). In contrast, viral infections showed marked seasonality, with winter peaks, consistent with respiratory virus circulation, and summer increases consistent with enterovirus/parechovirus activity (Figure 1 and 2A–2B). Adenovirus showed low counts but a relatively even distribution, supporting the concept of non-strict seasonality (Figure 2B). Fifty patients (6.1%) experienced a co-infection with two or more viral agents, whereas 27 bacterial infections (3.3%) were associated with a viral co-infection. Supplementary Table S1 reports the total burden of the identified viruses.

Viral etiologies declined in 2020–2021 and rebounded sharply in 2022, whereas the frequency of bacterial infections remained stable over time (Figure 3). SARS-CoV-2 became a major driver of viral detections after its emergence, contributing to the 2022 surge and to overall viral burden (Figures 3-4).

DISCUSSION

Although most febrile infants ≤90 days present with viral illnesses, 10–15% may develop a serious bacterial infection, predominantly UTIs and less frequently IBIs. In our cohort, SBIs accounted for 15% of cases, and IBIs showed a clear age dependency, peaking in infants ≤28 days. This finding reinforces the central role of age in risk stratification and supports a conservative diagnostic approach in neonates, regardless of viral detection (1-4,7,15,16). Approximately 10% of SBIs in our cohort occurred in the presence of a documented viral infection, consistent with multicenter PECARN data demonstrating that viral positivity reduces but not eliminate the risk of bacterial infection (Table 1) (1,7,16). Notably, this risk was largely driven by UTIs, confirming that urine testing should remain systematic in all febrile infants ≤90 days, irrespective of viral test results

(7,17). In our cohort, the 61–90-day age group showed fewer documented viral infections (46.7%) and a higher proportion of presumptive viral infections (34.7%) compared with younger infants. This pattern likely reflects a more selective diagnostic strategy in older infants, in whom, once a UTI has been excluded, the lower perceived risk profile may have resulted in less systematic viral testing.

A key finding of our study is the clear dissociation between viral and bacterial seasonality. Viral infections demonstrated marked seasonal variation, with winter peaks driven by respiratory viruses and summer predominance of enterovirus and parechovirus, particularly among neonates. In contrast, bacterial infections showed no consistent seasonal pattern and remained relatively stable across months and years. This epidemiological independence suggests that bacterial evaluation should not be relaxed during periods of high viral circulation.

The COVID-19 pandemic provided a natural experiment supporting this interpretation. While viral detections declined sharply during 2020–2021 and rebounded in 2022, bacterial infection rates remained comparatively stable over time (4,15,18-19). These findings reinforce the concept that bacterial risk in young febrile infants is largely age-dependent rather than season-dependent.

Among specific viral pathogens, SARS-CoV-2 accounted for a substantial proportion of viral detections after 2020 and was more frequent in infants aged 29–60 days (15,18-19). Although SARS-CoV-2–positive infants generally showed a favorable clinical course, UTIs remained possible, consistent with previous studies (11,15,19-20). Similar patterns have also been observed for influenza and RSV, indicating that in well-appearing infants >28 days old with reassuring clinical evaluations, management intensity may be tailored while maintaining screening for UTIs (20-24). Rhinovirus was frequently identified, both in isolated infections and co-infections, and did not appear to meaningfully modify bacterial risk, supporting prior evidence that its detection has limited discriminatory value in this age group (31). Enterovirus and parechovirus demonstrated the expected summer predominance and were particularly relevant in neonates, aligning with known epidemiological patterns (7,26-27).

Taken together, our findings suggest that viral diagnostics may provide useful etiological context, particularly in infants older than 28 days, but should be interpreted within structured clinical algorithms incorporating age, clinical appearance, urinalysis, and

inflammatory markers. Age remains the most robust determinant of invasive bacterial risk, while viral testing may support contextual decision-making without replacing bacterial evaluation.

Limitations

This study has several limitations. First, its retrospective single-center design may limit the generalizability of our findings, as viral circulation patterns, diagnostic strategies, and resource availability may differ across regions and healthcare systems. Second, changes in viral testing availability and strategy over time, particularly during and after the COVID-19 pandemic, may have introduced detection bias and contributed to underestimation of certain viral etiologies or co-infections. This led to missing data mainly related to presumptive viral infections, as viral testing was not performed systematically in all infants, particularly during the pandemic–when hospital policies limited PED testing primarily to SARS-CoV-2. Third, the use of broader multiplex viral panels was selectively based on clinical judgment, potentially influencing pathogen detection rate, particularly in infants aged 61-90 days. Fourth, the relatively small number of IBIs limited the ability to perform subgroup analyses, particularly for rare viral–bacterial co-infections.

Finally, the study focused on etiological distribution and did not systematically evaluate long-term clinical outcomes, limiting conclusions regarding the prognostic implications of viral positivity.

Despite these limitations, the large cohort size and extended observation period provide a robust epidemiological overview of febrile infants ≤90 days across pre-pandemic, pandemic, and post-pandemic phases.

CONCLUSION

In febrile infants ≤90 days, viral infections account for the majority of cases and display marked seasonal variability, whereas serious bacterial infections remain relatively stable and are strongly age-dependent. Although viral detection reduces the probability of concurrent bacterial infection, it does not eliminate it, particularly for urinary tract infections.

These findings support an age-driven, structured diagnostic approach in which viral testing provides contextual information but does not replace systematic bacterial evaluation, particularly in neonates. In this population, age remains the most reliable determinant of invasive bacterial risk, and clinical decision-making should prioritize this factor over seasonal epidemiology or viral positivity alone.

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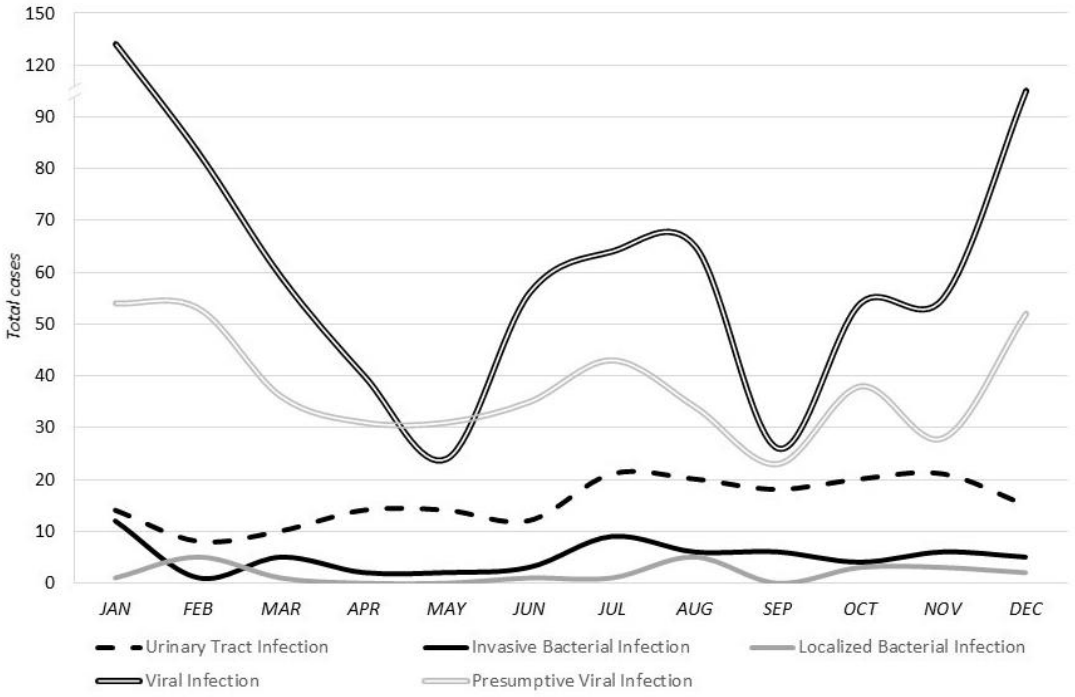

Figure 1. Monthly distribution of etiologic categories in febrile infants <90 days, showing bacterial infections (urinary tract infection, invasive bacterial infection and localized bacterial infection) and viral etiologies (documented and presumptive).

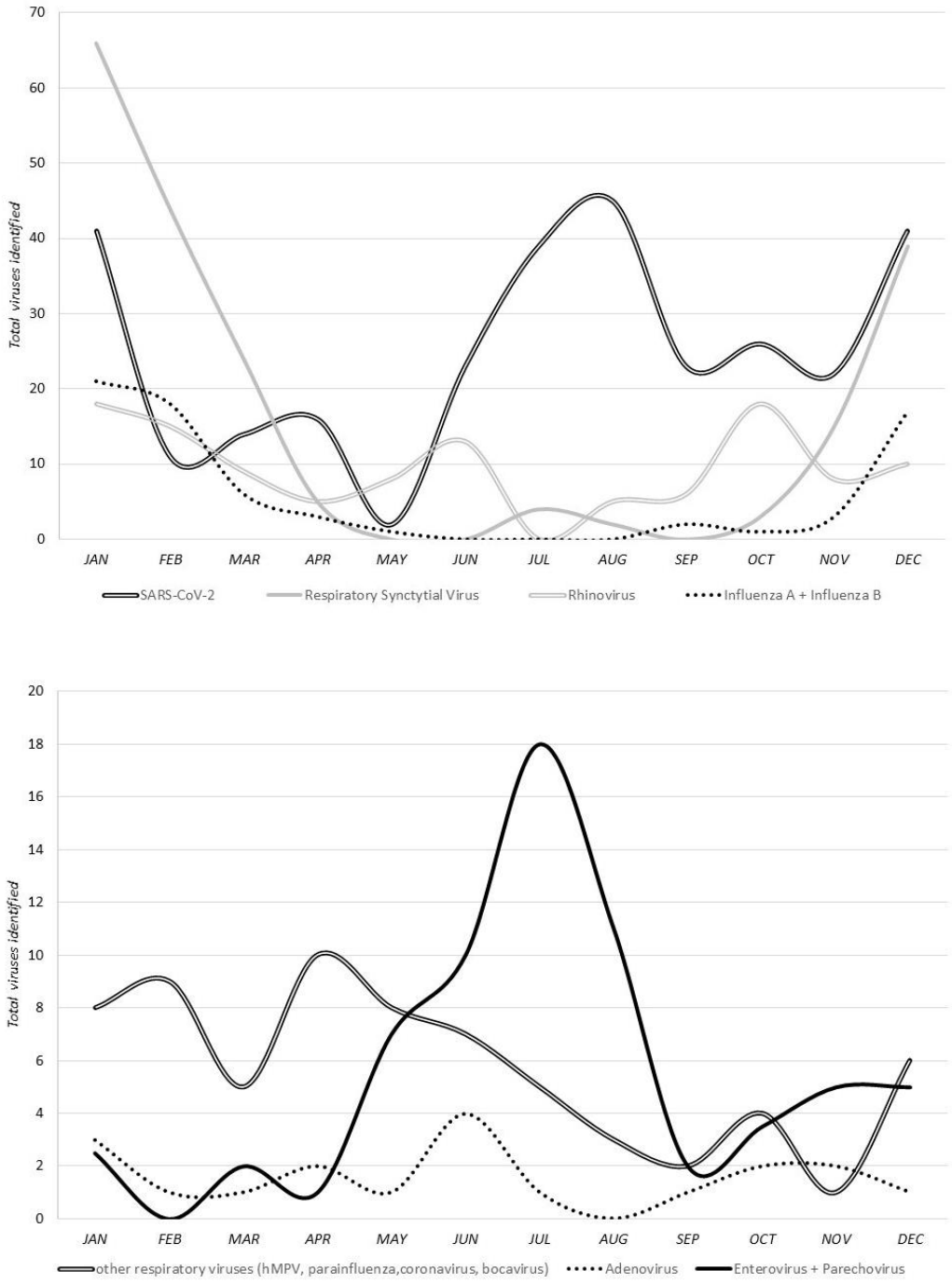

Figure 2. Panels are separated to improve readability and scaling **A.** Monthly distribution of the most frequent respiratory viruses identified in febrile infants <90 days. **B.** Monthly distribution of less frequent viruses identified in febrile infants <90 days. Panels are separated from Figure 3A to improve readability and scaling.

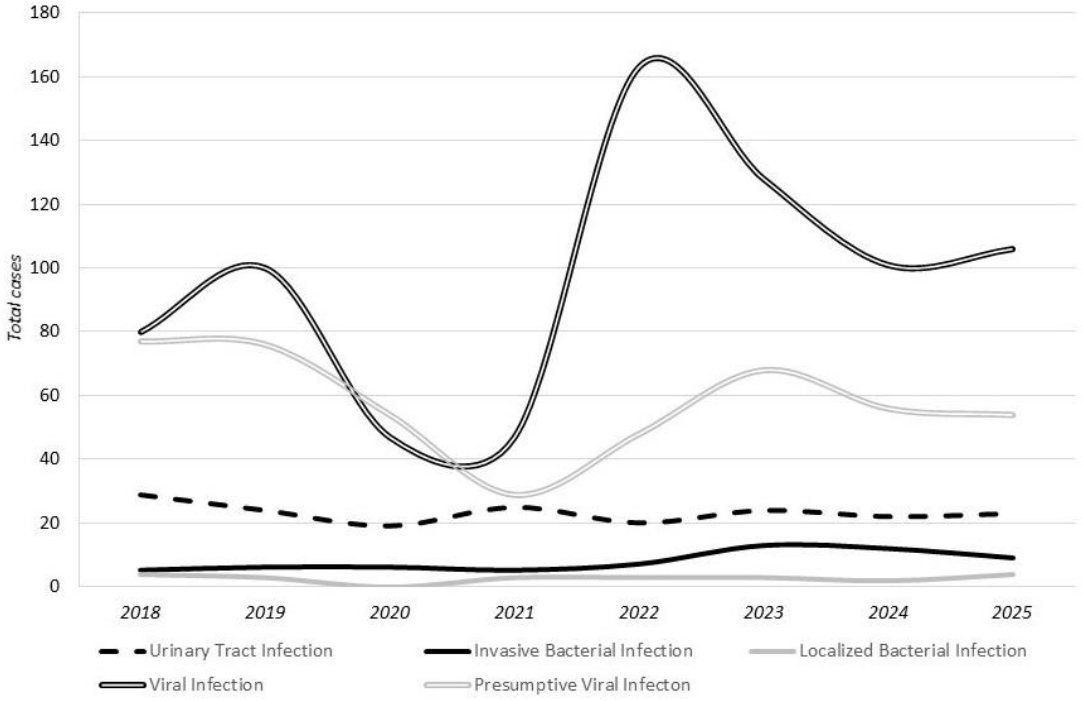

Figure 3. Annual distribution of etiologic categories in febrile infants <90 days (2018–2025). Categories include urinary tract infection, invasive bacterial infections (bacteremia and bacterial meningitis), localized bacterial infections, documented viral infections, and presumptive viral infections.

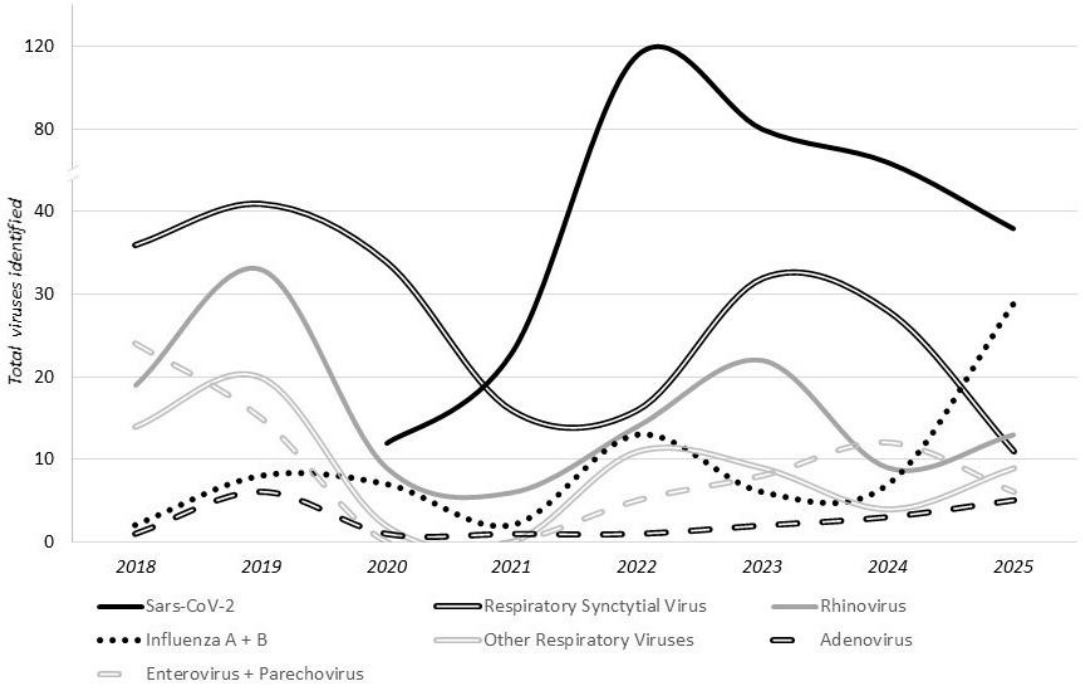

Figure 4. Annual trends of identified viral pathogens in febrile infants <90 days (2018–2025). The figure displays the annual number of detections for major respiratory viruses and other viral pathogens identified through point-of-care testing and/or RT-PCR according to local availability over the study period.

	Total patients (n = 1515)	≤ 28 days old patients (n = 345)	29-60 days old patients (n = 638)	61-90 days old patients (n = 532)	*p*
Sex, *male (%)*	912 (60.1)	198 (57.4)	394 (61.7)	320 (60.1)	0.4
Urinary Tract Infection, *yes (%)*	170 (11.2)	39 (11.3)	71 (11.1)	60 (11.3)	0.99
Urinary Tract Infection + Viral Co-infection, *yes (%)*	17 (1.1)	2 (0.6)	7 (1.1)	8 (1.5)	0.46
Invasive Bacterial Infection, *yes (%)*	56 (3.7)	24 (6.9)	23 (3.6)	9 (1.7)	**0.0002**
Invasive Bacterial Infection + Viral Co-infection, *yes (%)*	7 (0.5)	0 (0)	3 (0.5)	4 (0.7)	0.28
Localized Bacterial Infection, *yes (%)*	19 (1.3)	6 (1.7)	6 (1.0)	7 (1.3)	0.55
Localized Bacterial Infection + Viral Co-infection, *yes (%)*	3 (0.2)	2 (0.6)	0 (0)	1 (0.2)	0.22
Viral Infection, *yes (%)*	784 (51.7)	177 (51.3)	353 (55.3)	254 (47.7)	**0.0011**
Presumptive Viral Infection, *yes (%)*	459 (30.3)	95 (27.6)	175 (27.4)	189 (35.6)	**0.0011**

Table 1. Etiologic classification of febrile infants <90 days, overall and by age group. Categories include urinary tract infections, invasive bacterial infections, localized bacterial infections, documented viral infections, presumptive viral infections, and bacterial–viral co-infections.

	Total patients (n = 811)	≤ 28 days old patients (n = 181)	29-60 days old patients (n = 363)	61-90 days old patients (n = 267)	*p*
Sars-CoV-2	293 (36.2)	55 (30.4)	147 (40.5)	91 (34.0)	**0.048**
RSV	164 (20.3)	35 (19.4)	65 (17.9)	64 (23.9)	0.16
Rhinovirus	77 (9.5)	19 (10.5)	32 (8.8)	26 (9.7)	0.8
FluA	49 (6.1)	9 (4.9)	18 (5.0)	22 (8.6)	0.18
FluB	9 (1.1)	2 (1.1)	7 (1.9)	0 (0)	0.07
hMPV	25 (3.1)	5 (2.7)	10 (2.8)	10 (3.7)	0.74
Parainfluenza	22 (2.7)	3 (1.6)	10 (2.8)	9 (3.3)	0.54
Adenovirus	11 (1.4)	3 (1.6)	3 (0.8)	5 (1.9)	0.49
Enterovirus	46 (5.7)	21 (11.6)	20 (5.5)	5 (1.9)	**0.0001**
Parechovirus	12 (1.5)	8 (4.4)	4 (1.1)	0 (0)	**0.0005**
Bocavirus	2 (0.2)	1 (0.6)	1 (0.3)	0 (0)	0.50
Coronavirus	6 (0.7)	0 (0)	3 (0.8)	3 (1.1)	0.38
HHV-6	9 (1.1)	2 (1.1)	5 (1.4)	2 (0.7)	0.75
CMV	1 (0.1)	0 (0)	1 (0.3)	0 (0)	0.53
HSV-1	1 (0.1)	1 (0.6)	0 (0)	0 (0)	0.17
Rotavirus	5 (0.6)	1 (0.6)	3 (0.8)	1 (0.4)	0.76
Norovirus	2 (0.2)	1 (0.6)	1 (0.3)	0 (0)	0.5
Viral-Viral Co-infections	50 (6.1)	11 (6.1)	23 (6.3)	16 (6.0)	0.98
Bacterial-Viral Co-infections	27 (3.3)	4 (2.2)	10 (2.7)	13 (4.8)	0.21

Table 2. Distribution of identified viral etiologies and co-infections among febrile infants <90 days, stratified by age group. *Sars-CoV-2,* Severe Acute Respiratory Syndrome CoronaVirus 2*; RSV,* Respiratory Syncytial Virus*; FluA,* influenza A virus*; FluB,* influenza B virus*; hMPV,* human Metapneumovirus*; HHV-6,* Human Herpes Virus 6*; CMV,* Cytomegalovirus*; HSV-1,* Herpes Simplex Virus 1.

	Total patients (n = 811)	≤ 28 days old patients (n = 181)	29-60 days old patients (n = 363)	61-90 days old patients (n = 267)***	*p*
Sars-CoV-2	300 (37.0)	56 (30.9)	150 (41.3)	94 (35.2)	**0.046**
RSV	200 (24.6)	42 (23.2)	81 (22.3)	77 (28.8)	0.15
Rhinovirus	108 (13.3)	24 (13.2)	43 (11.8)	41 (15.3)	0.43
FluA	55 (6.8)	9 (4.9)	22 (6.0)	24 (8.9)	0.19
FluB	13 (1.6)	3 (1.6)	8 (2.2)	2 (0.7)	0.35
hMPV	28 (3.4)	5 (2.7)	11 (3.0)	12 (4.5)	0.51
Parainfluenza	27 (3.3)	4 (2.2)	11 (3.0)	12 (4.5)	0.38
Adenovirus	17 (2.1)	4 (2.2)	4 (1.1)	9 (3.3)	0.14
Enterovirus	59 (7.3)	26 (14.3)	27 (7.4)	6 (2.2)	**< 0.0001**
Parechovirus	13 (1.5)	9 (4.9)	4 (1.1)	0 (0)	**0.0001**
Bocavirus	3 (0.3)	1 (0.5)	2 (0.5)	0 (0)	0.47
Coronavirus	12 (1.5)	2 (1.1)	5 (1.3)	5 (1.9)	0.78
HHV-6	9 (1.1)	2 (1.1)	5 (1.3)	2 (0.7)	0.75
CMV	1 (0.1)	0 (0)	1 (0.3)	0 (0)	0.54
HSV-1	1 (0.1)	1 (0.5)	0 (0)	0 (0)	0.17
Rotavirus	7 (0.8)	1 (0.5)	5 (1.5)	1 (0.4)	0.35
Norovirus	2 (0.2)	1 (0.5)	1 (0.3)	0 (0)	0.50

Table S1. Total identified viral pathogens in febrile infants younger than 90 days, stratified by age group. This table reports the total number of viral detections, including viruses identified in single viral infections, viral–viral co-infections, and bacterial–viral co-infections. As individual patients could contribute more than one viral detection, totals exceed the number of virus-positive infants. *Sars-CoV-2,* Severe Acute Respiratory Syndrome CoronaVirus 2*; RSV,* Respiratory Syncytial Virus*; FluA,* influenza A virus*; FluB,* influenza B virus*; hMPV,* human Metapneumovirus*; HHV-6,* Human Herpes Virus 6*; CMV,* Cytomegalovirus*; HSV-1,* Herpes Simplex Virus 1.

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RESEARCH

Clinical course and peculiarities of Parechovirus and Enterovirus central nervous system infections in newborns: a single-center experience

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Abstract

Parechovirus (HpEV) and Enterovirus (EV) infections in children mostly have a mild course but are particularly fearsome in newborns in whom they may cause aseptic meningitis, encephalitis, and myocarditis. Our study aimed to describe the clinical presentations and peculiarities of CNS infection by HpEV and EV in neonates. This is a single-center retrospective study at Istituto Gaslini, Genoa, Italy. Infants aged ≤ 30 days with a CSF RTq-PCR positive for EV or HpEV from January 1, 2022, to December 1, 2023, were enrolled. Each patient's record included demographic data, blood and CSF tests, brain MRI, therapies, length of stay, ICU admission, complications, and mortality. The two groups were compared to identify any differences and similarities. Twenty-five patients (15 EV and 10 HpEV) with a median age of 15 days were included. EV patients had a more frequent history of prematurity/neonatal respiratory distress syndrome ($p = 0.021$), more respiratory symptoms on admission ($p = 0.012$), and higher C-reactive protein (CRP) levels ($p = 0.027$), whereas ferritin values were significantly increased in HpEV patients ($p = 0.001$). Eight patients had a pathological brain MRI, equally distributed between the two groups. Three EV patients developed myocarditis and one HpEV necrotizing enterocolitis with HLH-like. No deaths occurred.

Conclusion: EV and HpEV CNS infections are not easily distinguishable by clinical features. In both cases, brain MRI abnormalities are not uncommon, and a severe course of the disease is possible. Hyper-ferritinemia may represent an additional diagnostic clue for HpEV infection, and its monitoring is recommended to intercept HLH early and initiate immunomodulatory treatment. Larger studies are needed to confirm our findings.

What is Known:
- *Parechovirus and Enteroviruses are the most common viral pathogens responsible for sepsis and meningoencephalitis in neonates and young infants.*
- *The clinical course and distinguishing features of Parechovirus and Enterovirus central nervous system infections are not well described.*

What is New:
- *Severe disease course, brain MRI abnormalities, and complications are not uncommon in newborns with Parechovirus and Enteroviruses central nervous system infections.*
- *Hyper-ferritinemia may represent an additional diagnostic clue for Parechovirus infection and its monitoring is recommended.*

Keywords Parechovirus · Enterovirus · Newborns · Central nervous system · Viral infection

Abbreviations

CNS	Central nervous system
CSF	Cerebrospinal fluid
CRP	C-reactive protein
EV	Enterovirus
HLH	Hemophagocytic lymphohistiocytosis
HpEV	Human parechoviruses
IVIG	Intravenous immunoglobulins
NEC	Necrotizing enterocolitis
NICU	Neonatal intensive care units

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Extended author information available on the last page of the article

PICU Pediatric intensive care unit
PCR Polymerase chain reaction

Introduction

Human parechoviruses (HpEV) and Enteroviruses (EV) are small, single-stranded RNA viruses that belong to the family Picornaviridae [1, 2]. Both viruses have a typical seasonal distribution during the spring–summer months and are transmitted from person to person via fecal–oral or respiratory routes [1, 2]. Poliomyelitis is the most widely described example of a disease caused by an EV, but with the introduction of anti-polio vaccines in the 1950s, it has been almost eradicated [2]. The decrease in poliomyelitis has increased the recognition of the infection burden of the non-polio EV and HpEV, which share with poliovirus a characteristic central nervous system (CNS) tropism [1–5].

EV and HpEV infections are observed in all age groups, but mainly in children below 1 year of age [1, 2, 4]. Although infections in children are mostly asymptomatic or associated with mild disease, both EV and HpEV are particularly fearful in newborns because of the immaturity of their immune systems. In this age group, severe and sometimes fatal conditions can be observed, including aseptic meningitis, encephalitis, myocarditis, and acute hepatitis [2, 4, 6, 7]. These viruses may represent up to 20% of non-bacterial neonatal sepsis, although there is still a lack of their real incidence and impact on the pediatric population, especially in newborns [8].

Polymerase chain reaction (PCR) is the gold standard for diagnosing EV and HpEV infections and helps reduce the length of hospital stay and inappropriate antibiotic therapies. It can be performed on various tissue samples, including cerebrospinal fluid (CSF), blood, nasopharyngeal swabs, urine, and stool [9].

Previous reports have described the clinical presentation of EV and HpEV infections at different ages, and some authors have also attempted to differentiate between the two viruses, including proposing a scoring system for young infants [10]. However, definitive data on the clinical course and distinguishing features in newborns are not available [11, 12].

Following this, there are no actual recommendations for managing neonates with confirmed EV/HpEV infections and the decision to perform further investigations (CSF analysis, brain magnetic resonance imaging—MRI—and echocardiography) or specific treatment (i.e., intravenous immunoglobulin, antiepileptic drugs, etc.) is left to physicians.

The main guidelines on the management of febrile infants and infants focus on identifying patients at risk of severe or invasive bacterial infection. Furthermore, the widespread use of rapid viral PCR and multiplex respiratory viral testing is not yet standardized [13, 14].

Our study aimed to perform a critical analysis of the clinical features, blood and CSF tests, neuroradiological findings, treatment, and outcomes of 25 newborns with CNS infections due to EV or HpEV, first to better characterize the disease course improving the management of these patients, and second to highlight any significant difference between the two groups.

Materials and methods

We retrospectively collected data from the medical records of infants admitted to a tertiary children's hospital (IRCCS Istituto Giannina Gaslini, Genoa, Italy) from January 1, 2022, to December 1, 2023.

We included all patients younger than 30 days of age with a CSF reverse transcriptase real-time quantitative PCR positive for EV or HpEV.

Following the WHO disease outbreak news of May 31, 2023 [15] describing the incidence increase of severe neonatal infections from Echovirus 11, the Italian Ministry of Health issued an alert about this condition [16]. Starting from June 2023, all infants with EV-positive PCR on CSF were notified and sequenced.

Demographic data (age, sex, presence of older siblings, pathological perinatal history), symptoms on admission, blood tests, CSF examinations, MRI findings, and therapies administered were collected. Furthermore, outcome data (i.e., the need for admission in neonatal or pediatric intensive care units—NICU, PICU—the need for invasive or non-invasive ventilation, length of stay, occurrence of complications, and mortality) were gathered.

A comparison was made between patients with EV and HpEV infection to identify distinguishing features and similarities.

Kruskal–Wallis test or Mann–Whitney *U* test for continuous variables and chi-square or Fisher's exact test for categorical variables were used to assess differences between the groups. Statistical significance was set at $p < 0.05$, and all values were determined using two-tailed tests. All statistical analyses were conducted using IBM SPSS Statistics for Windows Version 21.0 (IBM Corp. Armonk, NY, USA).

Results

Twenty-five patients (15 EV, 10 HpEV) with an overall incidence of 3.7 EV/HpEV CNS infections per 10,000 pediatric Emergency Department admissions were included. The median age was 15.0 (range 8.0–22) days with a slight prevalence of males (56%). In both groups, the seasonal distribution showed a predominance of cases during the

summer months with 76% of cases occurring between May and September (Fig. 1).

Partial sequencing of the VP1 protein gene was performed in 6/15 EV infections (40%): 2 samples did not have sufficient material for sequencing, 2 were identified as coxsackievirus B2 and 2 were typed as echovirus 11 and 32, respectively.

The key data are summarized in Table 1 and 2.

Clinical and laboratory data

Fifteen patients (60%) had at least one older sibling and were equally distributed. Six children with EV infection had a history of prematurity (31–35 weeks of gestational age) and among them, two developed peri-partum respiratory distress syndrome (RDS) while this was not reported in patients with HpEV infection ($p = 0.021$). All patients were discharged home after birth and were readmitted to the hospital due to EV/HpEV infection. A substantial overlap of clinical features on admission was observed, although patients with EV infection showed more respiratory symptoms ($p = 0.012$). On the other hand, no differences were observed in the frequency of CNS involvement, in terms of drowsiness, irritability, and hypotonia.

Among blood tests, higher CRP values on admission ($p = 0.027$) were noticed in patients with EV infection. Both groups showed increased median ferritin levels that were significantly higher in patients with HpEV infection (3599 vs 891 ng/ml, $p = 0.001$). Furthermore, EV patients presented a higher CSF protein and cell count ($p = 0.002$ and $p = 0.016$, respectively).

Brain MRI findings

All patients underwent brain MRI during their hospital stay. Among them, nine patients (36%) had pathological findings, equally distributed between the two groups (4/10 HpEV and 5/15 EV patients). In particular, three patients with HpEV infection showed diffuse restriction of diffusion in the supratentorial periventricular white matter following the distribution of deep medullary veins, and one had reduced blood flow in the right transverse sinus, suggesting intracranial venous congestion (Fig. 2). Two EV patients showed abnormal signal of the ependymal wall of the lateral ventricles, whereas the remaining three EV presented multiple T2-hyperintense focal lesions in the periventricular white matter without contrast enhancement (Fig. 2).

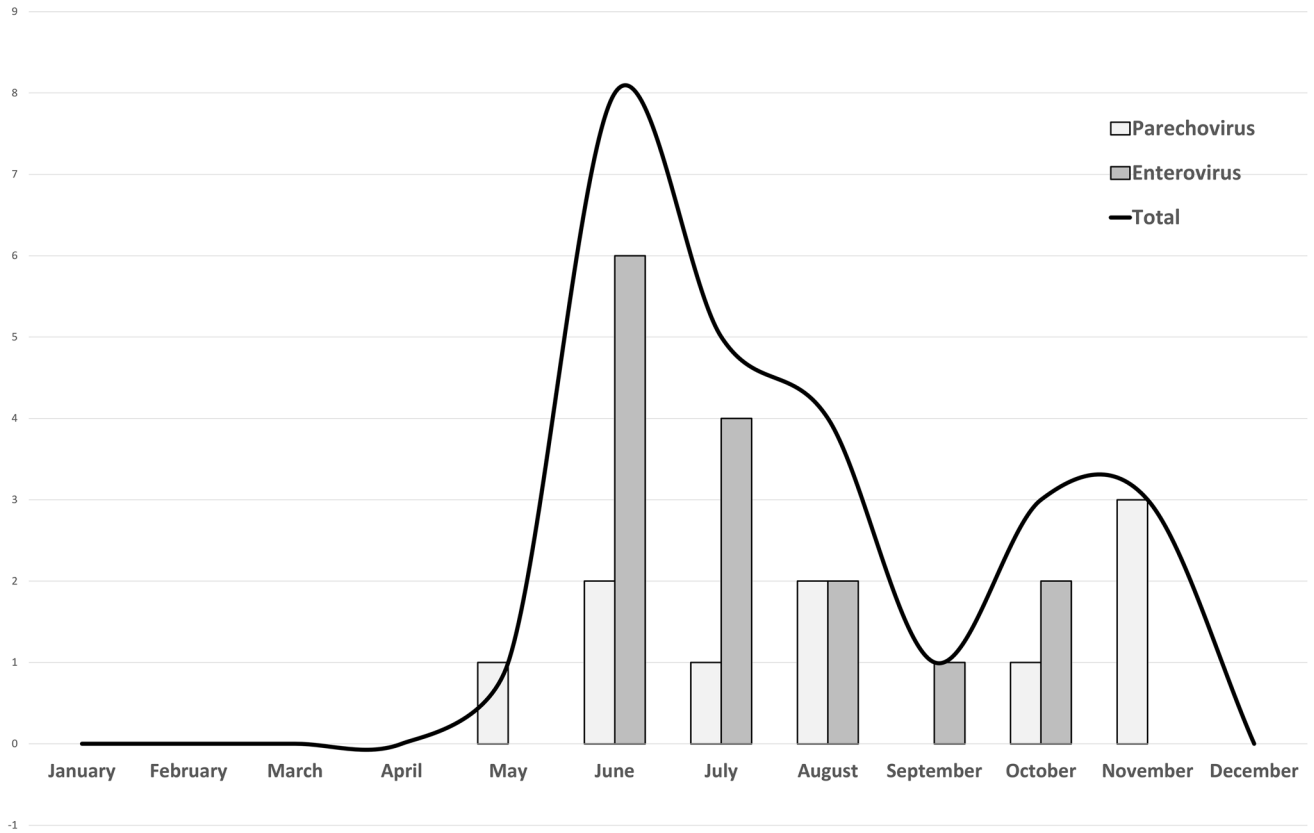

Fig. 1 Temporal distribution of cases of neonates with Enterovirus or Parechovirus central nervous system infection

Table 1 Demographic, clinical, MRI, therapy, and outcome data of newborns with Enterovirus or Parechovirus CNS infection

	Total $n=25$	**Parechovirus** $n=10$	**Enterovirus** $n=15$	***p***
Age, days, *median (IQR 25–75)*	15.0 (8.0–22.0)	15.5 (9.5–18.5)	14.0 (6.0–25.5)	0.75
Sex, *male (%)*	14 (56%)	8 (80%)	6 (40%)	**0.048**
Older sibling, *yes (%)*	15 (60%)	6 (60%)	9 (60%)	1
GBS-positive mother, *yes (%)*	3 (12%)	2 (20%)	1 (6.5%)	0.31
Comorbidities, *yes (%)*	6 (24%)	0 (0%)	6 (40%)	**0.021**
Poor clinical condition on arrival, *yes (%)*	12 (48%)	5 (50%)	7 (46.5%)	0.86
Fever, *yes (%)*	21 (84%)	10 (100%)	11 (73%)	0.074
GI symptoms, *yes (%)*	1 (4%)	0 (%)	1 (6.5%)	0.40
Respiratory symptoms, *yes (%)*	10 (40%)	1 (10%)	9 (60%)	**0.012**
CNS symptoms, *yes (%)*	21 (84%)	9 (90%)	12 (80%)	0.50
Skin rash, *yes (%)*	2 (8%)	0 (0%)	2 (13%)	0.22
Pathological brain MRI, *yes (%)*	9 (36%)	4 (40%)	5 (33%)	0.4
IV antibiotics, *yes (%)*	25 (100%)	10 (100%)	15 (100%)	1
IV acyclovir, *yes (%)*	15 (60%)	5 (50%)	10 (66.5%)	0.40
Immunomodulatory therapies, yes (%)	4 (16%)	3 (30%)	1 (6.5%)	0.11
NICU/PICU admission, *yes (%)*	11 (44%)	1 (10%)	10 (66.5%)	**0.005**
Mechanical ventilation, *yes (%)*	3 (12%)	1 (10%)	2 (13%)	0.80
HFNC/CPAP, *yes (%)*	8 (32%)	0 (0%)	8 (53%)	**0.005**
Length of stay, days, *median (IQR 25–75)*	11.0 (9.0–22.0)	10.0 (8.0–22.0)	11.0 (9.0–21.0)	0.49
Complications, *yes (%)*	4 (16%)	1 (10%)	3 (20%)	0.50
Myocarditis, *yes (%)*	3 (12%)	0 (0%)	3 (20%)	0.13
HLH-like disease, *yes* (%)	1 (4%)	1 (10%)	0 (0%)	0.21
Death, *yes (%)*	0 (0%)	0 (0%)	0 (0%)	\

Significant *p* values (<.05) are expressed in bold

GBS group B Streptococcus, *GI* gastro-intestinal, *CNS* central nervous system, *IV* intravenous, *NICU* neonatal intensive care unit, *PICU* pediatric intensive care unit, *HFNC* high flow nasal cannula, *CPAP* continuous positive airway pressure, *HLH* hemophagocytic lymphohistiocytosis

Table 2 Blood and cerebrospinal fluid test of newborns with Enterovirus or Parechovirus central nervous system infection

	Total $n=25$	**Parechovirus** $n=10$	**Enterovirus** $n=15$	***p***
CRP (mg/dl), *median (IQR 25–75)*	0.4 (0.0–1.1)	0.0 (0.0–0.0)	0.6 (0.2–1.5)	**0.027**
CRP > 2 mg/dl, *yes (%)*	3 (12.5%)	0 (0%)	3 (20%)	0.13
CRP max (mg/dl), *median (IQR 25–75)*	0.6 (0.0–3.7)	0.0 (0.0–0.8)	2.5 (0.0–4.4)	0.052
PCT (ng/ml), *median (IQR 25–75)*	0.3 (0.1–0.8)	0.5 (0.1–0.8)	0.2 (0.1–0.4)	0.39
PCT > 0,5 ng/ml, *yes (%)*	5 (20%)	3 (30%)	1 (6.5%)	0.11
WBC (cells/mm^3), *median (IQR 25–75)*	8530 (6600–11,120)	7985 (5780–9075)	8520 (7105–11,170)	0.42
ANC (cells/mm^3), *median (IQR 25–75)*	3860 (2390–6020)	3000 (2260–3930)	4270 (3340–6645)	0.19
ALC (cells/mm^3), *median (IQR 25–75)*	2660 (1460–3960)	2150 (1445–3845)	2660 (1640–3830)	0.69
ALC < 1,500 cells/mm^3, *yes (%)*	7 (28%)	4 (40%)	3 (20%)	0.27
Platelets (10^3/mm^3), *median (IQR 25–75)*	293 (234–384)	319 (248–373)	272 (230–411)	0.88
Hemoglobin (g/dl), *median (IQR 25–75)*	13.4 (12.3–16.0)	13.9 (12.8–15.1)	13.0 (11.7–17.1)	0.95
Fibrinogen (mg/dl), *median (IQR 25–75)*	302 (229–353)	229 (220–349)	326 (289–359)	0.21
Ferritin (ng/ml), *median (IQR 25–75)*	1274 (932–3595)	3599 (1623–5793)	891 (651.3–1017.5)	**0.001**
Albumin (mg/dl), *median (IQR 25–75)*	2771 (2,441–3,008)	3000 (2400–3010)	2769 (2577–2862)	0.22
CSF cells/mm^3, *median (IQR 25–75)*	22.0 (6.0–111.5)	4.5 (2.8–16.0)	45.0 (10.5–171.1)	**0.016**
CSF cells > 10 cells/mm^3, *yes (%)*	13 (52%)	2 (20%)	11 (73%)	**0.008**
CSF protein, mg/dl, *median (IQR 25–75)*	62.4 (49.0–103.3)	50.5 (43.0–56.5)	93.3 (53.6–112.7)	**0.002**
CSF protein > 45 mg/dl, *yes (%)*	19 (76%)	5 (50%)	14 (93%)	**0.012**

Significant *p* values (<.05) are expressed in bold

CRP C-reactive protein, *PCT* procalcitonin, *WBC* white blood cells, *ANC* absolute neutrophils count, *ALC* absolute lymphocyte count, *CSF* cerebrospinal fluid

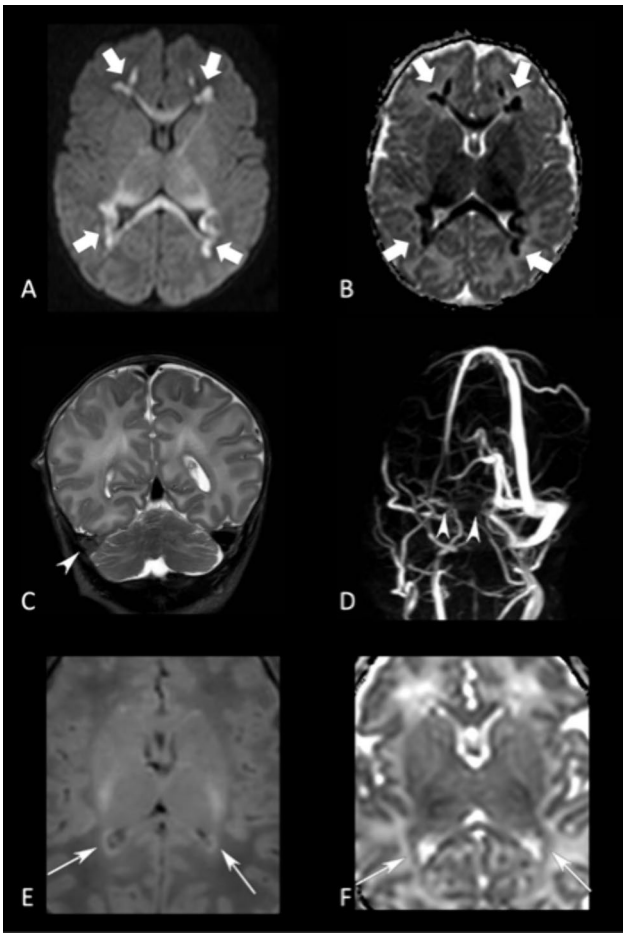

Fig. 2 **A** Axial diffusion-weighted MRI of the brain (repetition time ms/echo time ms, 8500/100; flip angle, 90°; *b*-value = 1000 s/mm^2) and **B** corresponding axial apparent diffusion coefficient map (8500/100; flip angle, 90°; *b*-value = 1000 s/mm^2) showing areas of restricted diffusion involving the bilateral supratentorial white matter (solid white arrows on all images) and corpus callosum in a newborn with Paraechovirus encephalitis. **C** Coronal T2-weighted image and **D** 3D-phase-contrast MR venography showing reduced blood flow in the right transverse sinus (white arrowheads on all images) of a neonate with Paraechovirus encephalitis. **E** Axial T1-weighted image and **F** axial apparent diffusion coefficient map (8500/100; flip angle, 90°; *b*-value = 1000 s/mm^2) showing T1 hyperintensity and mild restriction of diffusion of the ependymal wall of lateral ventricles (thin white arrows on all images) in a neonate with Enterovirus encephalitis and ventriculitis

Outcome and treatment

Patients with EV infection required more frequent admission to PICU/NICU (66% vs. 10%, $p = 0.005$) and non-invasive respiratory support (0% vs. 53%, $p = 0.005$). However, there were no significant differences in the need for intubation and median length of stay.

Complications were observed in four patients (16%): one child in the HpEV group had a severe course of disease with the development of Necrotizing Enterocolitis (NEC) and hemophagocytic lymphohistiocytosis (HLH)-like syndrome while three patients with EV infection developed myocarditis.

Empirical antibiotic therapy was started in all patients. Antiviral therapy with acyclovir was administered to 15 (60%) patients according to risk factors for HSV. Both treatments were stopped when CSF RT-PCR turned positive for EV or HpEV.

Overall, 4 (16%) patients received immunomodulatory therapy: anakinra and steroids were given to the HpEV patient with HLH-like syndrome, and anakinra alone was administered to other two HpEV patients following the concern related to increasing ferritin levels associated with hypofibrinogenemia in one case, and persisting fever in the other. In the EV group, one patient with acute myocarditis received intravenous immunoglobulins (IVIG) plus anakinra.

All patients were hospitalized and discharged without any evidence of short-term neurological sequelae. No deaths occurred.

Discussion

EV and HpEV are major causes of infection in children and may present with a variety of clinical manifestations [1–4, 6]. In neonates and young infants, EV and HpEV have been reported to cause sepsis-like illnesses and severe CNS infections. It has been reported that the younger the patient, the more severe the clinical manifestations [2, 4, 6, 7].

Evidence on neonates is usually based on small case series or case reports, so incidence data are not conclusive [6, 7]. Furthermore, available studies comparing EV and HpEV-infected neonates are limited [10, 17].

In our study, we observed a typical seasonal distribution in both groups, with most cases peaking during the spring–summer months.

In 15 of the 25 patients, an older sibling with a cold was reported, with no difference between the two groups, confirming that an older sibling may play an important role in transmission [8].

The clinical presentation of the two groups was overlapping, except for a higher prevalence of respiratory symptoms in patients with EV infection. This finding appears to be in contrast with other reports, where HpEV patients suffered more apneas and received more frequent oxygen administration compared to patients with EV infections [18]. A possible explanation may be found in the higher frequency of prematurity and neonatal respiratory distress syndrome in our EV group which may lead to greater respiratory vulnerability.

Interestingly, while many patients presented with irritability as an early symptom (21 patients, 84%), no patient suffered seizures, which has been reported as a common clinical feature [2, 4, 8].

We observed no differences in the length of hospital stay, although patients with EV showed a greater need for ICU admission and non-invasive ventilation, suggesting a more complicated clinical course.

This could be also explained by the concern related to the higher prevalence of prematurity among EV patients and the following need for closer monitoring.

Interestingly, the patient with echovirus 11-confirmed disease developed severe myocarditis and required ICU admission with a significant hospital stay of 22 days, confirming a more severe disease course in this subgroup of patients.

Especially within the last decade since genotyping is possible and widely used, it has become clear that not all genotypes are associated with the same rate of clinical manifestations [19, 20].

Unfortunately, there is currently no dedicated surveillance system for non-polio Enteroviruses in Italy, and we have typed only a limited number of patients in our series. However, a large European investigation referring to the years 2015–2017 revealed that Coxsackievirus A6 and Echovirus 30 were the most frequently detected EV types [21]. More recently, two outbreaks of Echovirus 30 were identified by an Italian survey [22].

Patients with EV infection showed slightly higher CRP values on admission although not reaching statistical significance considering values > 2 mg/dl or considering the highest value reached during hospitalization. As previously reported [23], these data support that repeated CRP values of < 2 mg/dl may allow the exclusion of bacterial infections in febrile infants. On the other hand, these cut-offs do not allow to predict the disease course in patients suffering from a viral infection which sometimes, as observed in our case series, can expose them to serious complications [13, 14].

The most striking data of our analysis was represented by the significant increase in ferritin levels observed in all our patients with HpEV infection. Hyper-ferritinemia may suggest a viral sepsis-like state or the development of a secondary HLH since both conditions have been described in association with HpEV [18, 24]. Following this concern, in our series, we treated three HpEV-infected patients with anakinra, although only one patient developed 4 criteria out of 5 required for the diagnosis of HLH [25].

Our data suggest that the elevation of ferritin may represent an additional diagnostic clue for HpEV infection. In addition, monitoring of ferritin levels and other HLH parameters is recommended during hospitalization to early intercept the development of HLH and initiate appropriate immunomodulatory treatment. However, further studies are needed to assess which ferritin levels may suggest the impending evolution to secondary HLH.

Children in the EV group had higher CSF cell counts and protein levels. This is likely due to greater blood–brain barrier damage secondary to an increased cytokine expression in children with CNS infection by EV compared with HpEV [11, 12]. The clinical symptoms of these two viruses are similar, but their pathogenicity and interactions with the immune system and CSN may differ. EV seems to infect a variety of CNS parenchymal cells, including neurons, whereas HpEV CNS infection is not well understood and may affect leptomeningeal blood vessels [11, 12].

However, our data on pleocytosis should be interpreted with caution considering the low case numbers within the subgroups of our series and that cerebrospinal fluid white blood cell count reference values in neonates and young infants are still debated [26]. Furthermore, some authors in other seasons have reported a lack of CSF pleocytosis especially in children under 3 months [27], and a more common CSF pleocytosis in older children [28]. Similarly, CSF protein levels seem to be age-dependent and decrease with each month of life. More research is needed to confirm our findings [29]. We observed no significant differences in the brain MRI pathological findings between the two groups. Three HpEV patients and three EV patients had extensive involvement of the bilateral white matter, which is the most common lesion described in this type of viral CNS infection [30, 31].

The remaining two EV patients showed inflammatory signs involving the ventricular ependyma, and one patient with HpEV infection presented reduced blood flow in the transverse sinus with an increased risk of venous thrombosis. All of these findings suggest that the majority of lesions occurring in the pediatric brain during viral infections are more likely related to inflammatory mechanisms contributing to endothelial dysfunction, rather than to direct infection of brain tissues [8, 11, 32, 33]. Also for these reasons, brain MRI plays a key role in the estimation of parenchymal involvement and the prediction of neurodevelopmental outcomes, but it does not show sufficient specificity to distinguish among the different viral etiologies [31, 33–35].

Although the overall outcome of our population was good, a non-negligible number of newborns in our series (16%) developed severe complications.

The HpEV patient with HLH also developed a NEC. It has been anecdotally reported that HpEV infection may be associated with the development of NEC in both preterm and full-term infants [36] and that abdominal distension may be a striking feature of HpEV infection presentation, often mimicking acute abdomen [37].

Our case reinforces the need for careful evaluation of possible abdominal involvement in children with HpEV infection. Furthermore, we suggest that prompt initiation of intravenous antibiotic therapy and early discontinuation of enteral feeding should be considered in case of abdominal distension and/or feeding intolerance.

Three patients with EV infection developed acute myocarditis. Myocarditis is a rare but well-described complication of EV and occasionally of HpEV infection, associated with

serious long-term complications and death in all age groups, particularly in infants, with a mortality rate of 30–50% [38, 39]. Recently, an increase in EV-associated myocarditis has been reported [40]. Dosage of myocardial necrosis enzymes should be routinely assessed in children with documented EV and HpEV infection, especially in unwell neonates, or in the presence of inappropriate persistent tachycardia.

There are currently no Food and Drug Administration-approved therapies for EV or HpEV infections in any age group. Considering that in the majority of cases, EV and HpEV are responsible for self-limiting or mild disease, the standard treatment is represented by supportive care.

However, as observed in our study, in case of complications or life-threatening infections, the administration of immunomodulatory drugs may be required [4].

IGIV has been used for severe neonatal EV infections but with only anecdotal evidence for HpEV [41].

Anakinra, an interleukin-1-receptor antagonist, has shown efficacy in the treatment of secondary HLH [42] and EV-associated HLH [43]. Moreover, its use has been also reported in a preliminary experience treating pediatric acute fulminant myocarditis, following the presumed central role of IL-1 pathways in pathogenesis [44]. While not yet demonstrated by controlled trials in HLH, its safety profile appears to be good even when administered at high doses (5–10 mg/Kg) and intravenously [45].

Finally, specific antiviral therapies have been proposed such as pleconaril, a capsid inhibitor that prevents the virus from attaching to cell receptors, and pocapavir, a selective anti-enteroviral agent, but data on widespread use in pediatrics are limited [1, 2, 7, 8].

Little is known about the prognosis and long-term effects of EV and HpEV CNS infections in children. Some authors suggest long-term neurological impairment, while other case series do not describe differences between children with viral meningitis and control groups [1, 4, 31]. We do not have sufficient data to provide a conclusion, but we believe that follow-up of patients with CNS viral infections should be recommended, especially if it occurs in the first months of life.

This study has several limitations. First, the retrospective design and the single center setting of the study. Second, we typed the viruses isolated from CSF in a limited number of cases, so we cannot be precise about a causal relationship between the subtypes of EV and HpEV and their neurovirulence. Furthermore, the small sample size limits the power of statistical analysis and the generalization of our findings.

In conclusion, non-polio EV and HpEV are prevalent worldwide and are important causes of neonatal morbidity and mortality. Most infections may be asymptomatic, but severe cases with CNS involvement and fearful complications such as myocarditis or HLH are possible, representing a global threat. Although it is difficult to distinguish between the two viruses based on clinical symptoms alone, EV neonates seem to have more respiratory involvement. Hyper-ferritinemia may represent an additional diagnostic clue for HpEV infection, and its monitoring is recommended to intercept HLH early and initiate immunomodulatory treatment helping reduce morbidity and mortality. Further larger studies are needed to confirm our findings.

Authors' contributions All authors contributed to the study conception and design. GB designed data collection instruments, coordinated data collection, and critically reviewed and revised the manuscript. TB drafted the initial manuscript, designed data collection instruments, performed the statistical analysis, and critically reviewed and revised the manuscript. MM, MP, MR, IB, DT, AP, EF, AM, and JT collected data, conducted the initial analysis, and critically reviewed and revised the manuscript. EP, LAR, and AM supervised the study and critically reviewed and revised the manuscript. All authors read and approved the final manuscript.

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Data availability Data sets generated during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval and consent to participate This study was conducted in accordance with the Helsinki Declaration. According to Italian legislation, the study did not need ethical approval, as it was a purely observational retrospective study on routinely collected anonymous data. Furthermore, it was not possible to request informed consent for participation in the study, given the nature of the study. In any case, consent to completely anonymous use of clinical data for research/epidemiological purposes is requested during clinical routine at the time of admission/diagnostic procedure.

Competing interests The authors have no relevant financial or non-financial interests.

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Human parechovirus infection in neonates and young infants: Clinical features, diagnostic challenges, and neurodevelopmental outcomes

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ABSTRACT

Human parechoviruses (HPeVs) are increasingly recognized as viral pathogens in neonates and young infants. Among the 18 identified genotypes, HPeV-3 is most strongly associated with severe disease during early infancy, including sepsis-like illness and central nervous system (CNS) infection. Clinical recognition can be challenging because typical laboratory features of CNS infection are frequently absent: cerebrospinal fluid (CSF) pleocytosis is often lacking, and inflammatory markers are usually normal or only mildly elevated. Severe cases may present with marked hyperferritinemia and systemic inflammatory activation resembling hemophagocytic lymphohistiocytosis. This narrative review summarizes current evidence on the epidemiology, transmission, pathophysiology, clinical manifestations, diagnostic approaches, management, and long-term outcomes of HPeV infection in neonates and young infants, with particular focus on HPeV-3. Evidence from recent cohort studies and outbreak investigations highlights the strong age dependence of severe disease, which predominantly affects infants younger than three months. Neuroimaging frequently reveals characteristic white matter injury, and the presence of seizures or extensive diffusion restriction on MRI is associated with increased risk of adverse neurodevelopmental outcomes. Diagnosis relies primarily on molecular detection using reverse-transcription polymerase chain reaction, with blood testing often providing higher diagnostic yield than CSF alone. Although no specific antiviral therapy is currently available, establishing a virological diagnosis has important clinical implications, including antimicrobial stewardship and identification of infants requiring neurodevelopmental follow-up. Despite increasing recognition of HPeV-3 infection, important knowledge gaps remain regarding mechanisms of brain injury, optimal diagnostic strategies, and long-term outcomes. Further prospective studies are needed to guide clinical management and inform preventive strategies.

1. Introduction

Human parechoviruses (HPeVs) are small, non-enveloped, single-stranded RNA viruses belonging to the genus *Parechovirus* within the family *Picornaviridae* [1]. Since their molecular reclassification in the early 2000s, HPeVs have emerged as clinically relevant pathogens, particularly in neonates and young infants [2]. Eighteen genotypes have been identified, with genotype-specific differences in epidemiology and disease severity [3,4]. While HPeV infections are common in childhood and often asymptomatic or mild, HPeV-3 is recognized as a leading cause of sepsis-like illness and central nervous system (CNS) infection in infants younger than 3 months [5]. The widespread adoption of molecular diagnostics has substantially increased detection rates, revealing a previously underrecognized burden of disease in this vulnerable age group. HPeV infection in early infancy presents distinctive diagnostic challenges: CNS involvement frequently occurs without CSF pleocytosis, and inflammatory markers are often low or only mildly elevated [6,7]. These atypical features may delay recognition and prolong empirical

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antibiotic therapy. In addition, marked hyperferritinemia and features of systemic inflammatory activation—mimicking hemophagocytic lymphohistiocytosis (HLH)—have been described in severe HPeV-3 infection [8].

Epidemiologically, HPeV demonstrates seasonal and cyclical outbreak patterns, with biennial peaks reported in multiple temperate regions [2]. The 2022 resurgence of cases in the United States, which prompted a health advisory from the Centers for Disease Control and Prevention, highlighted the public health importance of this pathogen and reinforced the need for improved surveillance and diagnostic awareness [9,10].

Despite increasing recognition, important knowledge gaps remain regarding pathogenesis, optimal management, and long-term outcomes. No specific antiviral therapy is currently available, and data on immunomodulatory treatment are limited. Emerging evidence suggests that severe HPeV-3 infection, particularly when associated with white matter injury on MRI, carries a significant risk of adverse neurodevelopmental sequelae [11,12].

This narrative review summarizes current evidence on the epidemiology, transmission, clinical features, laboratory findings, diagnostic approaches, management, prevention, and long-term outcomes of HPeV infection in neonates and young infants, with a particular focus on HPeV-3.

2. Methods

This narrative review was conducted through a structured search of PubMed, Embase, and Scopus for articles published between January 2005 and December 2025. Search terms included "human parechovirus," "HPeV-3," "neonate," "infant," "meningoencephalitis," "sepsis-like illness," "white matter injury," and "neurodevelopmental outcome." We prioritized original research articles, prospective and multicenter cohort studies, and systematic reviews focusing on neonates and infants younger than 3 months. Case reports were included when addressing rare complications (e.g., myocarditis, HLH-like presentations). Reference lists of selected articles were screened to identify additional relevant studies. Given the narrative nature of the review, no formal quality assessment or meta-analytic synthesis was performed.

3. Epidemiology and transmission

3.1. Age distribution and incidence

Human parechovirus infection predominantly affects neonates and young infants, with severe disease occurring mainly during the first months of life [5,13]. HPeV-3–associated sepsis-like illness and CNS infection are most frequent in infants younger than 3 months, with a marked concentration of cases in the neonatal period [5,14].

During the 2022 resurgence in the United States, a multicenter investigation identified over 120 infants with laboratory-confirmed infection across 11 states [9,10]. Infants with severe neurologic disease had a significantly lower median age at presentation (13.9 days) compared with those with non-severe illness (30 days) [9]. Similar age distributions have been reported in European and Australian cohorts, with median presentation consistently within the first month of life [12,15]. Population-based surveillance further identifies enterovirus and HPeV as leading viral causes of pediatric meningitis, with HPeV contributing disproportionately to early infancy [13,15].

3.2. Genotype distribution and seasonality

Although 18 HPeV genotypes have been identified, severe neonatal disease is overwhelmingly associated with HPeV-3 (≈85% of cases) [3]. In contrast, genotypes such as HPeV-1, −4, and −6 are more commonly detected in older infants and are typically associated with mild respiratory or gastrointestinal illness [5].

More recently, the emergence of HPeV-5–associated central nervous system infections in children has been reported, although these cases appear to be associated with milder clinical manifestations compared with HPeV-3 infection [16].

HPeV-3 infections demonstrate variable seasonality influenced by geographic location and climate. In temperate regions such as the United States, Europe, and Australia, summer peaks with biennial epidemic cycles are frequently observed, often in even-numbered years [13,17]. In contrast, subtropical regions like Hong Kong exhibit autumn-winter seasonality (September-January) [18]. Although most cases in temperate climates occur between July and December, year-round circulation with sporadic detections has been documented, and the overall epidemiology and seasonality patterns remain incompletely understood [19].

3.3. Global seroprevalence and geographic distribution

HPeV infection is nearly universal in childhood, but genotype-specific immunity differs substantially. Seroprevalence of HPeV-1 exceeds 90% in adults, reflecting widespread early exposure. In contrast, HPeV-3 seroprevalence remains low (10–13%) in many regions, particularly in Europe [20,21].

This limited population immunity results in reduced maternal neutralizing antibody transfer to neonates, contributing to the disproportionate burden of severe HPeV-3 disease in early infancy. Infants with severe infection consistently demonstrate absent or low neutralizing titers (≤1:16) at disease onset, supporting the protective role of maternal antibodies [14,22].

3.4. Transmission and co-infections

HPeV is transmitted via fecal–oral and respiratory routes, facilitating household and healthcare spread [13]. Severe neonatal disease appears closely linked to maternal immunity patterns; mothers of affected infants frequently test positive for HPeV-3 and may have low neutralizing titers, suggesting exposure without effective antibody transfer [20,22,23].

Bacterial co-infection is uncommon in hospitalized infants with severe HPeV infection [9]. When co-pathogens are detected, viral agents are more frequent than invasive bacterial organisms [5]. The rarity of concurrent serious bacterial infection underscores the importance of considering HPeV in acutely unwell young infants, particularly when inflammatory markers are low.

4. Pathophysiology

The mechanisms underlying neurologic involvement in HPeV infection are incompletely understood but likely involve a combination of viral neurotropism, systemic inflammation, and developmental vulnerability of the neonatal brain.

4.1. Neuronal tropism and viral entry

HPeV-3 demonstrates distinctive neurotropism compared with other genotypes. Unlike HPeV-1 and several other types, HPeV-3 lacks the arginine–glycine–aspartic acid (RGD) motif in the VP1 capsid protein that mediates integrin-dependent cellular entry [24]. This suggests the use of alternative, integrin-independent receptors, potentially contributing to enhanced endothelial or neural tropism. Structural analyses support genotype-specific receptor interactions and may partly explain the neurovirulence of HPeV-3 [25].

Following initial mucosal replication, high serum viral loads lead to primary viremia and systemic dissemination [13,19]. Neonatal immune immaturity, attenuated type I interferon responses, and limited maternal neutralizing antibodies facilitate hematogenous spread [14,20]. The predominance of blood PCR positivity compared with CSF supports the

concept that systemic viral burden precedes or exceeds CNS invasion [26].

4.2. Blood–brain barrier and white matter vulnerability

Central nervous system invasion is thought to occur predominantly through hematogenous spread. The neonatal blood–brain barrier exhibits increased physiological permeability, which may facilitate viral translocation [5,27]. In addition, elevated systemic cytokines (IL-6, TNF-α, IFN-γ, IL-18) documented during HPeV-3 infection may further disrupt endothelial integrity and increase barrier permeability [28].

This cytokine-mediated endothelial dysfunction may partly explain the frequent observation of severe neurologic symptoms despite minimal CSF pleocytosis. Experimental neonatal models suggest that systemic inflammation and oxidative stress can induce microglial activation, impair oligodendrocyte maturation, and selectively injure premyelinating oligodendrocytes, supporting a dual-pathway model of injury combining hyperinflammation and white matter vulnerability [8,11,12].

Recent experimental data using human brain organoid models further suggest that neurologic injury may correlate more strongly with the host innate inflammatory response than with direct viral neurocytopathic effects, supporting the hypothesis that immune-mediated mechanisms contribute substantially to brain injury in HPeV infection [29].

5. Clinical manifestations

5.1. General presentation

In neonates and young infants, HPeV infection exhibits a wide range of clinical symptoms, from mild feverish conditions to severe sepsis-like illness and meningoencephalitis (Fig. 1). The most common symptoms include fever, significant irritability, poor feeding, tachycardia, and rash. The combination of fever, intense irritability, and a red rash has led to the description of affected infants as “hot, red, angry babies” [5,30]. HPeV-3 is closely linked to severe systemic illness that mimics bacterial sepsis [9]. Among hospitalized infants, fever, feeding difficulties, neurological symptoms (such as irritability or seizures), and rash are the most commonly reported signs [5,12,13]. Although leukopenia with

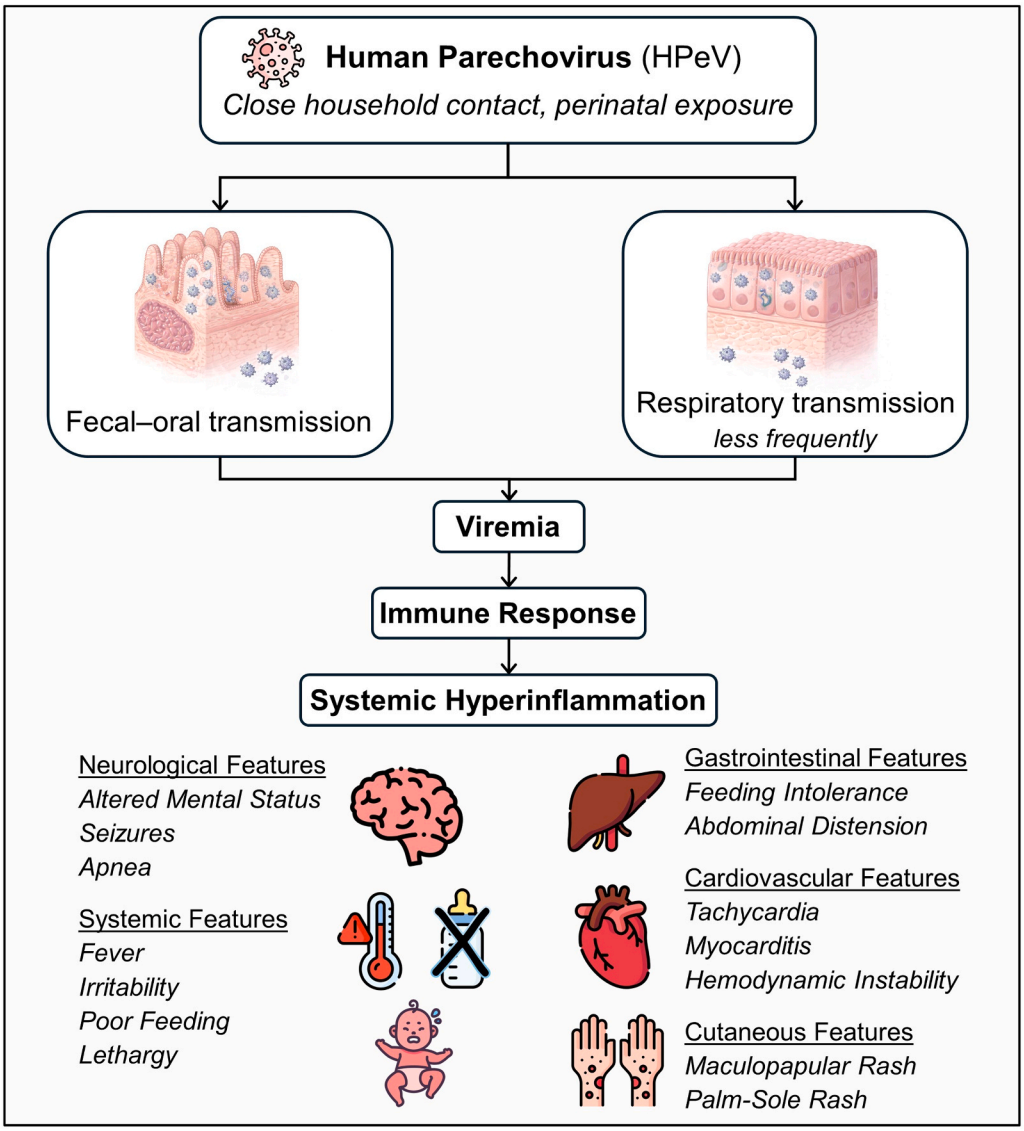

Fig. 1. Clinical spectrum of human parechovirus infection in neonates and young infants. Human parechovirus infection may present with a wide clinical spectrum ranging from mild febrile illness to severe sepsis-like disease and meningoencephalitis. Common findings include fever, marked irritability, rash, feeding difficulties, and gastrointestinal symptoms; severe cases may present with seizures and cardiorespiratory instability.

high fever has been proposed as a diagnostic clue, no single clinical feature reliably distinguishes HPeV-3 infection from other viral or bacterial causes of sepsis in young infants [5,13,30–32].

5.2. Cutaneous manifestations

Cutaneous involvement is common, particularly in HPeV-3–associated disease. A maculopapular rash typically develops within the first few days after fever onset and frequently involves the distal extremities, with characteristic erythema of the palms and soles (acral distribution) [33]. The rash is usually transient and not associated with mucosal involvement. Observational studies have shown a strong association between HPeV-3 infection, rash, and neurological symptoms such as seizures, reinforcing the diagnostic value of skin findings in the appropriate clinical context [34].

5.3. Gastrointestinal and abdominal manifestations

Gastrointestinal involvement represents an integral component of the clinical spectrum. While HPeV-1 is commonly associated with mild gastroenteritis in older children, HPeV-3 infection in neonates frequently includes prominent abdominal manifestations [35]. Abdominal distension is a particularly characteristic feature and may be accompanied by feeding intolerance and irritability. In some cases, gastrointestinal symptoms are sufficiently marked to mimic acute surgical conditions, including necrotizing enterocolitis (NEC) or intestinal obstruction (volvulus), potentially leading to unnecessary surgical consultation [36]. Recognition of the typical clinical and laboratory profile of HPeV infection—fever, rash, abdominal distension, leukopenia (often lymphopenia), and low or only mildly elevated inflammatory markers—may help avoid unnecessary invasive investigations and interventions (Figs. 1 and 2).

5.4. Sepsis-like illness

HPeV-3 is a well-recognized cause of severe sepsis-like illness in neonates. Affected infants may present with hypothermia or hyperthermia, apnea, and hemodynamic instability requiring fluid resuscitation or inotropes (reported in up to 28% of severe cases) [5,9,13,17,30]. Compared with infants with uncomplicated disease, those with severe sepsis-like illness tend to present at an earlier postnatal age and are more likely to have been born preterm [5,9,17].

5.5. Neurological manifestations

Neurological involvement is a major feature of severe HPeV infection, occurring in approximately 30% of hospitalized infants [9]. Clinical seizures are the most common neurological manifestation (reported in up to 57% of severe cases) and often occur early in the disease course [11,12]. Other neurological features include altered level of consciousness, central hypotonia, "startle" myoclonus, and persistent irritability. The presence of seizures is an independent predictor of white matter injury on MRI [12]. Infants with neurological involvement are typically younger at presentation and more likely to exhibit systemic signs of severe disease, including apnea and cardiovascular instability [9,11,12]. The clinical presentation frequently overlaps with that of enterovirus infection, underscoring the importance of specific virological testing [15,31,32].

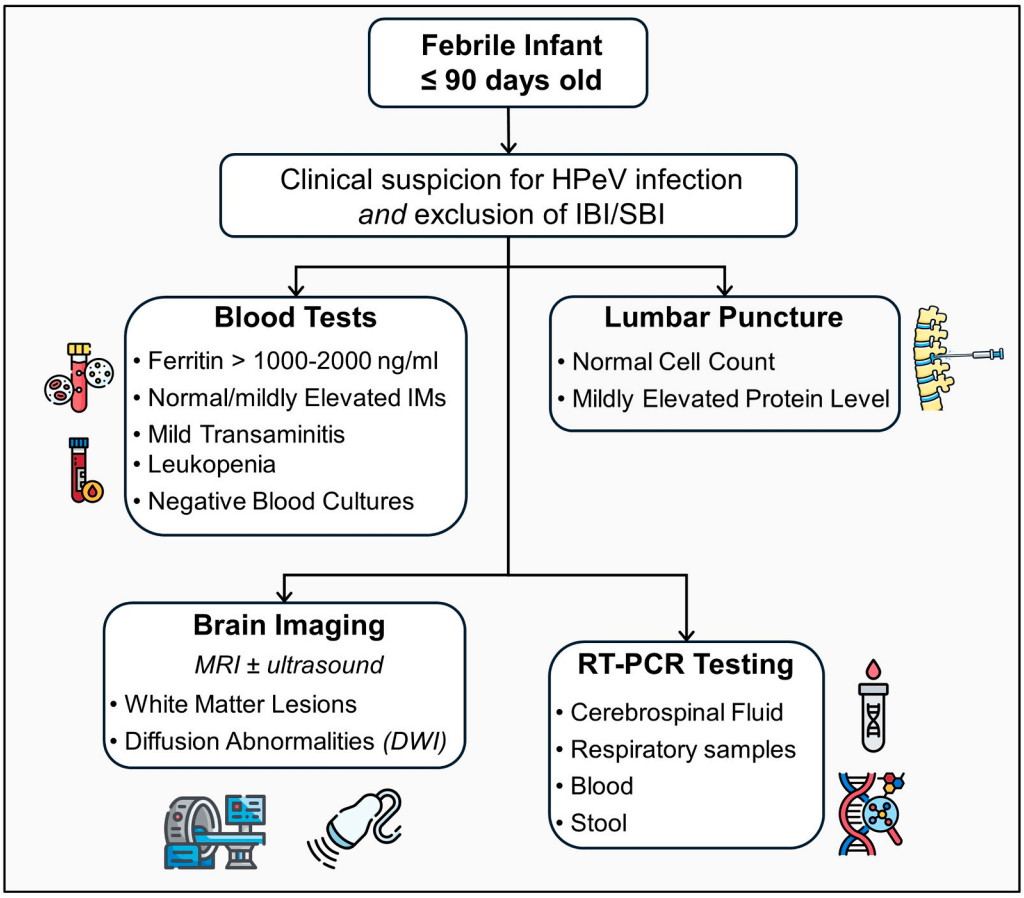

Fig. 2. Diagnostic pattern of human parechovirus infection. Typical clinical and laboratory features include fever, irritability, rash, leukopenia or lymphopenia, and normal or mildly elevated inflammatory markers. Cerebrospinal fluid pleocytosis is often absent despite central nervous system involvement. Diagnosis relies on RT-PCR, with blood testing often providing higher sensitivity than cerebrospinal fluid.

5.6. Other organ involvement

Although HPeV predominantly targets the central nervous system and leads to systemic inflammatory conditions in young infants, it can also affect other organ systems. Myocarditis, though rare, has been documented in isolated case reports and should be considered a potentially life-threatening complication in severely ill infants [37]. Hepatic involvement, indicated by elevated transaminase levels, is frequently observed in severe cases [38].

6. Laboratory findings

6.1. Cerebrospinal fluid (CSF) findings: the diagnostic paradox

A key feature of HPeV infection is the common absence of cerebrospinal fluid (CSF) pleocytosis, even in infants with clear signs of meningoencephalitis. Numerous studies have shown that HPeV can be present in CSF despite normal leukocyte counts and unremarkable biochemical parameters [12,13,30,39–41]. In cohort studies of infants with HPeV-positive CSF, most did not exhibit pleocytosis, emphasizing that a normal CSF white blood cell count should not rule out HPeV infection or determine the necessity for molecular testing [39,41]. Compared to enterovirus infection, HPeV infection is linked to significantly lower CSF leukocyte counts and similar protein levels [39,42]. CSF indices in infants with HPeV infection often resemble those in infants without central nervous system infection, highlighting the importance of pathogen-specific molecular diagnostics over traditional CSF parameters [13,40].

6.2. Hematologic and inflammatory markers

Peripheral blood abnormalities are common in infants with HPeV infection and may provide useful diagnostic clues. Leukopenia, particularly lymphopenia, is frequently observed, especially in HPeV-3 infection, and occurs more often than in enterovirus infection. Several comparative studies have shown significantly lower white blood cell and lymphocyte counts in HPeV-infected infants compared with both enterovirus-infected patients and uninfected controls [15,32]. Thrombocytopenia has also been reported, particularly in severe cases [8].

Markers of bacterial inflammation, including C-reactive protein and procalcitonin, are typically normal or only mildly elevated in HPeV infection. This laboratory profile may help differentiate HPeV from bacterial sepsis and, to a lesser extent, from enterovirus infection in young infants presenting with sepsis-like illness (Table 1) [8,32].

6.3. Hyperferritinemia and HLH-like features

Marked hyperferritinemia has emerged as a characteristic laboratory feature of HPeV-3 infection in neonates and young infants [8,43]. Ferritin concentrations frequently exceed 1000 ng/mL and typically peak during the second half of the first week of illness [8,43]. This temporal pattern suggests that ferritin measurement during the acute phase may aid in the recognition of HPeV-3 infection in febrile infants.

In a subset of patients, HPeV-3 infection is associated with clinical and laboratory features resembling hemophagocytic lymphohistiocytosis, including cytopenias, hyperferritinemia, elevated transaminases, lactate dehydrogenase, and coagulation abnormalities [8,9]. Although most infants recover with supportive care, rare cases may meet criteria for HLH and require targeted immunomodulatory treatment [31]. These findings suggest that severe HPeV-3 infection may trigger a dysregulated inflammatory response contributing to systemic illness.

6.4. Cytokine profiles

Limited studies investigating cytokine responses in HPeV-3 infection have demonstrated distinct inflammatory profiles compared with enterovirus infection [27,44,45]. Elevated serum levels of proinflammatory cytokines and chemokines, including interleukin-6, tumor necrosis factor–α, interferon-γ, and interleukin-18, have been reported during the acute phase of illness [27,44,45]. In contrast, cytokine concentrations in CSF are typically low or undetectable, a finding that may partially explain the absence of CSF pleocytosis despite neurological involvement [27,44,45].

These observations support the concept that HPeV-3–associated disease is driven by a systemic hyperinflammatory response rather than by prominent intrathecal inflammation.

7. Neuroimaging findings

Neuroimaging is essential for the diagnosis, management, and prognostic assessment of infants with human HPeV encephalitis. While cranial ultrasound (cUS) often shows increased periventricular

Table 1
Comparative clinical and laboratory features of human parechovirus type 3 (HPeV-3), enterovirus, and bacterial sepsis in young infants.

Feature	Human Parechovirus-3 (HPeV-3)	Enterovirus (Non-Polio)	Bacterial Sepsis / Meningitis
Peak Age	< 3 months (esp. neonates <28 days)	< 1 year (broad range)	< 1 month (GBS, E. coli)
*Seasonality**	Biennial peaks (Summer/Autumn)	Annual peaks (Summer/Autumn)	None (Year-round)
Clinical "Gestalt"	"Hot, Red, Angry Baby": Extreme irritability, pain on handling, flushed skin	Lethargy, fever, but often less "painful" irritability	Lethargy, poor perfusion, "toxic" appearance
Rash	Palmar/Plantar Erythema, maculopapular, distal extremities	Generalized maculopapular or petechial	Rare (except *N. meningitidis* - petechial/purpuric)
White Blood Cell Count	Leukopenia / Lymphopenia (<1000/mm^3)	Normal or mild Lymphocytosis	Leukocytosis or Neutropenia
CRP / Procalcitonin	Normal or mildly elevated	Normal or mildly elevated	High / Markedly elevated
Ferritin	Marked Hyperferritinemia (>1000–2000 ng/mL)	Normal or mildly elevated	Variable
CSF Pleocytosis	Absent / Minimal (Paradoxical normal CSF in encephalitis)	Present (often >10–100 cells/mm^3)	Marked (>100–1000 s cells/mm^3, neutrophilic)
Neuroimaging (MRI)	White Matter Injury: "Sunburst" diffusion restriction (DWI), corpus callosum	Gray Matter / Brainstem: Substantia nigra, pontine lesions	Meningeal enhancement, abscess, infarcts, hydrocephalus
Primary Specimen	Blood (Serum/Plasma) > CSF > Stool	CSF > Stool > Blood	Blood & CSF (Culture)
Seizure Risk	High (~30–50% in severe cases)	Variable (depends on genotype, e.g., EV-A71)	High in meningitis
Long-term Outcome	Risk of CP, white matter injury, developmental delay	Generally good (except severe EV-A71/D68)	Risk of hearing loss, global delay, hydrocephalus

Note: This table summarizes the distinct patterns useful for differential diagnosis in the acute setting. While overlap exists, HPeV-3 presents a unique profile characterized by the dissociation between severe clinical irritability and a lack of cerebrospinal fluid pleocytosis. **Abbreviations:** CSF, cerebrospinal fluid; WBC, white blood cell count; CRP, C-reactive protein; MRI, magnetic resonance imaging; DWI, diffusion-weighted imaging; CP, cerebral palsy. Data synthesized from: Evans et al. [9], Shah et al. [12], Olijve et al. [5], and Britton et al. [30].
*the reported seasonal patterns refer primarily to studies conducted in temperate regions and may not be generalizable to all geographic areas

echogenicity, it is less sensitive than Magnetic Resonance Imaging (MRI), which is the gold standard for identifying characteristic white matter injury [45–48]. Specifically, Diffusion-Weighted Imaging (DWI) reveals a pathognomonic radiating "sunburst" pattern of restricted diffusion in the periventricular and deep white matter, typically with frontoparietal predominance [49–52] (Fig. 3). The sources highlight that while the corpus callosum, internal capsules, and external capsules are frequently involved, the basal ganglia and infratentorial structures are usually spared, helping to differentiate HPeV from hypoxic-ischemic encephalopathy (HIE) [53]. Regarding management, early recognition of these imaging patterns provides critical diagnostic direction. It prompts specific HPeV PCR testing, which is often not included in routine viral panels, and may prevent extensive, unnecessary metabolic or genetic workups, resulting in significant time and cost savings [50].

8. Diagnostic approaches

A schematic overview of the diagnostic pattern is shown in Fig. 2.

8.1. Molecular diagnosis and available assays

The diagnosis of HPeV infection relies on molecular detection techniques, primarily reverse-transcription polymerase chain reaction (RT-PCR) [13,19,40]. Given the nonspecific clinical presentation, virological confirmation is essential [54]. During the 2022 outbreak, the CDC recommended HPeV testing for infants presenting with unexplained fever, sepsis-like illness, or neurological manifestations [9,40,41].

Most PCR assays target the highly conserved 5′-untranslated region (5′UTR) for virus detection, while the more variable VP1 and VP3 structural regions are used for genotyping [12,55]. Optimal assay sensitivity should approach 400 copies/mL in CSF to ensure detection of all HPeV genotypes [12]. Both singleplex and dual-target assays (detecting enterovirus and parechovirus simultaneously) have been developed and validated for clinical use [55].

Currently available molecular diagnostic approaches include singleplex HPeV-specific RT-PCR assays, dual-target enterovirus/parechovirus assays, and multiplex syndromic PCR panels as summarized in Table 2. Singleplex assays, often used in reference laboratories, provide high analytical sensitivity and may allow genotyping, but typically require specific test requests and are not universally available. Dual-target assays enable simultaneous detection of enterovirus and parechovirus on a single platform, reducing the risk of missed diagnoses when only enterovirus testing is ordered [55].

Multiplex syndromic panels, particularly meningitis/encephalitis arrays, enable rapid (~1 h) and simultaneous detection of multiple pathogens, including HPeV [56]. However, these platforms are restricted to CSF samples and may have lower sensitivity compared with blood-based testing in HPeV infection.

Critically, parechovirus detection relies predominantly on laboratory-developed tests, with only one FDA-cleared multiplex assay currently available (the FilmArray ME Panel) [57]. Many commercial multiplex meningoencephalitis panels do not include HPeV targets, which may contribute to underdiagnosis and create a false sense of security when results are negative. Clinicians must verify whether HPeV is included in their institution's panel before interpreting negative results.

8.2. Specimen selection and diagnostic yield

Selection of appropriate specimens is critical for optimal diagnostic sensitivity. Several studies have demonstrated that viral loads in blood (serum/plasma) are often significantly higher than in CSF, particularly in the early phase of infection. Accordingly, testing of whole blood or serum substantially improves diagnostic yield and should be performed in addition to CSF testing whenever possible [24,30].

Optimal timing for blood sampling is near symptom onset, with HPeV RT-PCR remaining positive with robust Ct values until at least day 5 of symptoms [58]. Importantly, HPeV is routinely detected in CSF despite normal white blood cell counts; therefore, testing should not be restricted based on CSF parameters [12]. Stool and nasopharyngeal swabs are also highly sensitive but may reflect prolonged viral shedding rather than acute invasive disease, limiting their specificity in the acute setting [53,54].

8.3. Diagnostic pitfalls and missed diagnoses

Despite increasing recognition, HPeV infection remains frequently underdiagnosed in routine clinical practice due to several critical pitfalls.

A major diagnostic pitfall occurs when clinicians order only enterovirus PCR and assume a negative result excludes all picornavirus infections. Parechoviruses belong to a different genus within the Picornaviridae family and require specific nucleic acid amplification testing—standard enterovirus assays do not detect parechovirus [57]. This misconception leads to substantial underdiagnosis, particularly since HPeV and enterovirus cause clinically indistinguishable presentations in young infants (fever, sepsis-like illness, meningitis). Clinicians may discontinue diagnostic workup after receiving a negative enterovirus PCR result, missing the parechovirus diagnosis entirely [12].

Another critical diagnostic challenge is the failure to test multiple specimen types, particularly blood in addition to CSF. Exclusive reliance on CSF testing may result in reduced diagnostic yield, as viral loads are often higher in blood [12,58]. Furthermore, the absence of CSF pleocytosis should not preclude HPeV testing, as CNS infection frequently occurs without inflammatory changes in the CSF [12].

8.4. Clinical impact and integration into febrile infant management

Although no specific antiviral therapy is available for HPeV infection, establishing a virological diagnosis carries important clinical implications. Confirmation of HPeV may support early discontinuation of empirical antibacterial therapy once bacterial cultures remain negative, reduce unnecessary diagnostic procedures, and potentially shorten hospital length of stay [9,30,32,40,41,54]. From a public health standpoint, improved diagnostic recognition facilitates surveillance and outbreak detection [13,40,41]. Clinically, identification of HPeV — particularly in infants with neurological involvement — also informs prognostic assessment and the need for structured neurodevelopmental follow-up [9,11,12].

The gradual shift from pathogen-specific testing (requiring clinician suspicion) to syndromic multiplex panels represents a major advance that is likely to improve detection rates and reduce diagnostic delays. Integration of HPeV testing into routine diagnostic algorithms for febrile neonates facilitates earlier recognition and supports antimicrobial stewardship.

Current febrile infant risk-stratification tools (Rochester, Step-by-Step, PECARN) are primarily designed to identify invasive bacterial infection and do not explicitly incorporate HPeV or other viral pathogens into their algorithms [59–61]. The role of routine HPeV testing remains debated. On one hand, systematic molecular detection may improve antibiotic stewardship and aid in identifying infants at risk for neurological sequelae. On the other, routine PCR testing of blood and cerebrospinal fluid raises concerns regarding incidental viral detection, given that parechovirus shedding may persist for weeks and that PCR positivity does not invariably indicate clinically significant invasive disease [13,34].

Interpretation of CSF positivity is particularly challenging because HPeV CNS infection frequently occurs in the absence of pleocytosis [12,32]. While multiple cohort studies confirm true encephalitis despite normal CSF cell counts, low-level viral detection could theoretically reflect transient viremia rather than established parenchymal infection [24,32,35]. Clinical correlation and, when indicated, brain MRI remain essential to distinguish invasive disease from incidental detection.

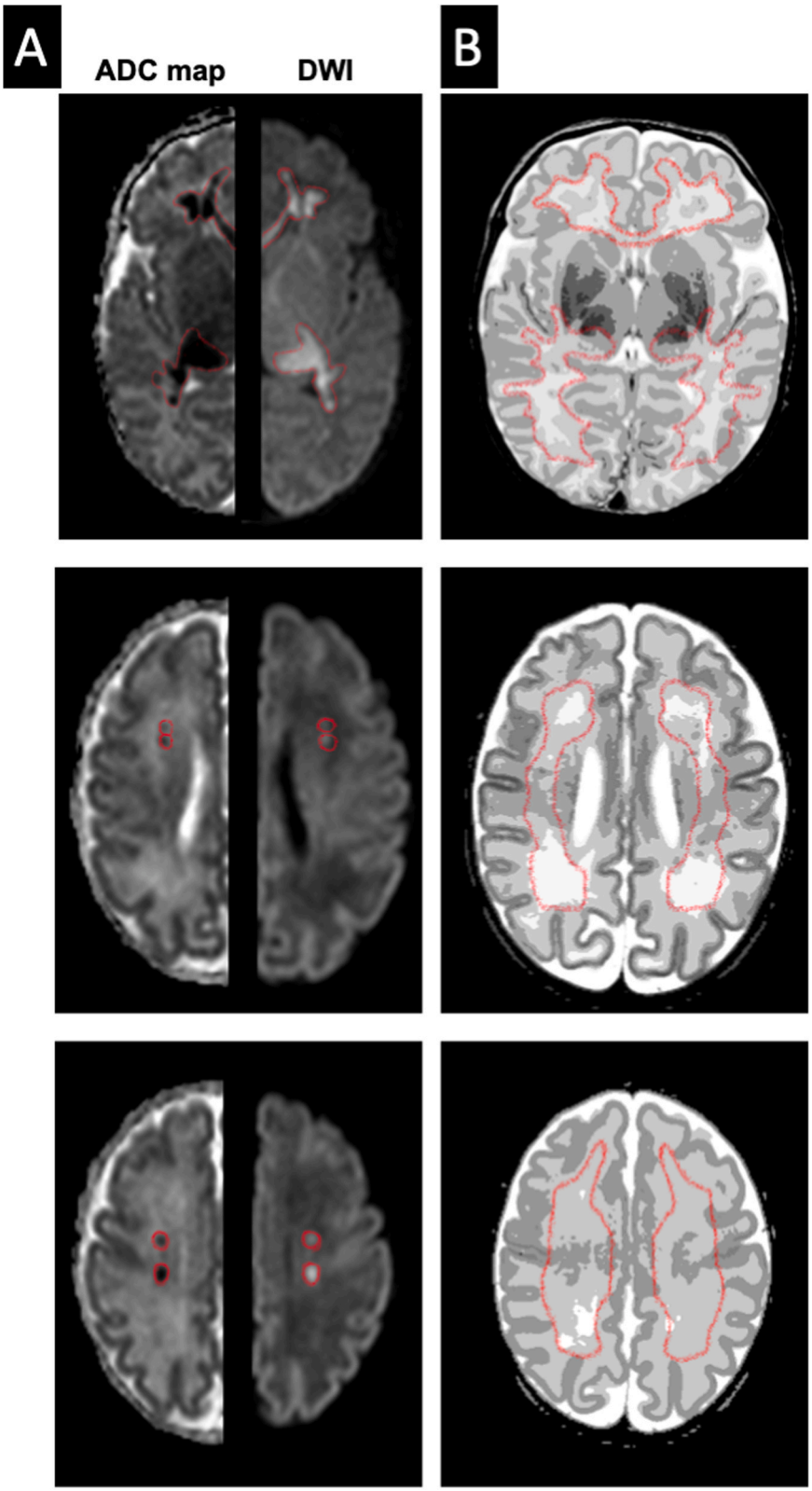

Fig. 3. Neuroimaging features of neonatal parechovirus infection. (A) Diffusion-weighted imaging (DWI) and apparent diffusion coefficient (ADC) maps showing areas of restricted diffusion (highlighted by red circles), consistent with acute white matter injury. The lesions are symmetrically distributed and predominantly affect the periventricular regions and deep white matter tracts, in keeping with the characteristic imaging pattern of neonatal parechovirus infection. (B) Schematic representation of the typical distribution of white matter lesions, predominantly involving the periventricular white matter, corona radiata, and centrum semiovale.

Table 2
PCR-based diagnostic assays for human parechovirus: overview of currently used approaches.

Test Type	Platform/Assay	Target(s)	Specimen Types	Key Features
Singleplex PCR	LDTs	HPeV 5′UTR	CSF, blood, stool	Most commonly used; requires specific clinical suspicion and ordering
Dual-target PCR	Various LDTs	EV + HPeV 5′UTR	CSF, blood	Simultaneous detection; reduces missed diagnoses
Multiplex Syndromic Panels	FilmArray Meningitis/ Encephalitis Panel	Multiple CNS pathogens including HPeV	CSF	Only FDA-cleared multiplex panel including HPeV; syndromic approach
Multiplex Syndromic Panels	Other commercial panels	Various pathogens (most exclude HPeV)	CSF, blood	Most do not include HPeV; clinicians must verify panel contents

Abbreviations: CSF, cerebrospinal fluid; EV, Enterovirus; HPeV, human parechovirus; LDTs, Laboratory-developed tests; RT-PCR, reverse-transcription polymerase chain reaction.

In light of these diagnostic challenges and limitations, a pragmatic approach to testing is essential in clinical practice. For infants < 6 months presenting with fever, sepsis-like illness, or neurological symptoms:

1. Both enterovirus AND parechovirus testing should be ordered reflexively
2. Both CSF AND blood specimens should be tested when possible
3. Testing should proceed regardless of CSF white blood cell count
4. A negative enterovirus PCR should never be interpreted as excluding picornavirus infection
5. Clinicians must verify whether their institution's multiplex panel includes HPeV before interpreting negative results

9. Management and treatment

9.1. Current therapeutic approaches

At present, no approved antiviral therapy exists for HPeV infection, and management remains primarily supportive. Treatment focuses on careful monitoring and management of complications [13,19,30]. Early identification allows for the optimization of supportive care and the avoidance of unnecessary interventions. Supportive management includes hydration, temperature control, and treatment of metabolic disturbances. Infants with severe disease may require admission to intensive care for cardiorespiratory support. Seizures should be promptly treated; they may be refractory and require multiple anticonvulsants [12,13,30].

9.2. Antimicrobial stewardship

Empirical antibacterial therapy is commonly initiated in febrile neonates. Confirmation of HPeV infection provides a crucial opportunity for early discontinuation of antibiotics and acyclovir, potentially reducing antibiotic exposure, hospital length of stay, and associated costs [13,32,54].

9.3. Intravenous immunoglobulin (IVIG)

Intravenous immunoglobulin (IVIG) has been used empirically in severe HPeV infection, particularly in cases with myocarditis or fulminant sepsis [25,37]. However, evidence supporting its efficacy is limited to case reports. Crucially, the efficacy of IVIG depends on the neutralizing antibody titers in the donor pool. Given the low seroprevalence of HPeV-3 in adults, commercial IVIG preparations may lack sufficient neutralizing activity against circulating strains [20,24]. Routine use cannot be recommended, though it may be considered on a case-by-case basis in life-threatening presentations.

9.4. Investigational therapies

Pleconaril, a capsid-binding agent effective against some enteroviruses, has shown no meaningful activity against HPeV-3 due to structural differences in the viral capsid binding pocket [62]. Monoclonal antibodies targeting HPeV capsid proteins have demonstrated in vitro activity but are not clinically available [25].

10. Long-term outcomes and follow-up

10.1. Neurodevelopmental outcomes

Long-term outcome data following HPeV infection remain limited but increasingly suggest a significant risk of neurodevelopmental sequelae, particularly after severe HPeV-3 infection with CNS involvement. Prospective cohorts indicate that up to 55% of infants with HPeV encephalitis develop neurodevelopmental deficits by 12 months, including cerebral palsy, central visual impairment, and gross motor delay [11,63–67]. Even in cohorts with less severe initial presentations, concerns regarding motor and problem-solving skills persist in ~28% of children at 3–4 years of age [14,63–67]. Importantly, neurodevelopmental deficits have been described even in infants who appeared neurologically normal at hospital discharge [11,63,64,66,67].

10.2. Correlation with neuroimaging findings

The prognostic role of neuroimaging is complex. In severe cases, acute white matter injury can evolve into cystic leukomalacia, severe gliosis, or significant volume loss, which often correlates with adverse outcomes like cerebral palsy or learning disabilities [51]. However, some studies also note that the severity of acute imaging findings does not always correlate perfectly with long-term neurodevelopment; some infants with extensive diffusion restriction demonstrate normal outcomes at short-term follow-up [68,69]. Conversely, infants with milder clinical presentations often have entirely normal imaging and favorable prognoses [53]. Therefore, while early MRI can outline the extent of injury to aid in discharge planning, long-term clinical follow-up remains necessary.

10.3. Outcomes after non-neurological severe disease

Data on outcomes following severe systemic HPeV infection without overt neurological involvement are scarce. Limited evidence suggests that infants presenting with sepsis-like illness who recover clinically may still be at risk for subtle neurodevelopmental sequelae, possibly due to the effects of systemic hyperinflammation and cytokine-mediated injury on the developing brain [13,30,64]. The extent of white matter damage in clinically mild cases remains unknown, and it is possible that some children will have neurological abnormalities leading to subtle deficits later in life [13,63,64].

10.4. Hearing and sensory outcomes

Unlike congenital viral infections such as CMV, sensorineural hearing loss has not been consistently associated with HPeV. Routine

audiologic follow-up beyond standard newborn screening is not universally recommended but may be considered in cases of severe meningoencephalitis [15].

10.5. Mortality

Although most infants with HPeV infection recover, mortality has been reported, particularly in cases with severe white matter abnormalities and HPeV-3 infection [70,71]. The largest study assessing case fatality rates found that approximately 4% (18/426) of infant deaths in autopsy specimens were attributable to HPeV [70]. In the 2022 U.S. outbreak, 2 infants with severe neurologic HPeV disease died during admission; no infant with mild disease died [9].

11. Follow-up recommendations

Given the potential for delayed sequelae and the discordance between short-term clinical appearance and long-term outcomes, structured neurodevelopmental follow-up is strongly recommended for all infants with confirmed HPeV infection who experienced severe disease, seizures, or abnormal MRI [11,12,30]. Follow-up programs should include standardized developmental assessments at 6, 12, and 24 months, with particular attention to motor function, visual development, and language acquisition. Early referral to rehabilitation services is advised when delays are identified. Infants with meningitis and no parenchymal involvement on MRI have been shown to have good clinical outcomes and may require less intensive follow-up [13,40].

12. Key research priorities

As highlighted throughout this review, several key knowledge gaps remain in the understanding and management of HPeV infection in early infancy. The clinical role of routine HPeV testing in the evaluation of febrile infants requires clarification, and prospective multicenter studies are needed to determine whether systematic incorporation of HPeV PCR into diagnostic algorithms improves outcomes and reduces unnecessary investigations.

Another important area concerns antimicrobial stewardship. Rapid viral diagnostics may facilitate earlier discontinuation of empirical antibiotic therapy, but interventional studies are required to confirm whether this strategy safely reduces antibiotic exposure and healthcare utilization.

Further research is also needed to better understand the mechanisms of brain injury in HPeV infection, particularly the relative contributions of direct viral cytotoxicity and dysregulated systemic inflammation. Finally, large longitudinal cohorts with standardized neuroimaging and neurodevelopmental assessments, together with expanded global surveillance, are essential to define long-term outcomes, protective immunity, and the true global burden of disease.

13. Conclusions

Human parechoviruses are increasingly recognized as important causes of sepsis-like illness and central nervous system infection in neonates and young infants. Although disease severity varies across genotypes, HPeV-3 remains most strongly associated with severe neurological involvement during early infancy. The distinctive clinical and laboratory profile of HPeV infection—particularly the frequent absence of cerebrospinal fluid pleocytosis—poses diagnostic challenges and underscores the central role of molecular testing in the evaluation of febrile infants.

While most infections are self-limited, severe cases may be associated with white matter injury and an increased risk of neurodevelopmental sequelae. Improved diagnostic awareness, integration of viral testing into clinical algorithms, and structured follow-up of high-risk infants will be essential to optimize clinical management and better define the long-term impact of HPeV infection.

CRediT authorship contribution statement

Andrea Moscatelli: Writing – review & editing, Visualization, Validation, Supervision. **Elio Castagnola:** Writing – review & editing, Visualization, Validation, Supervision. **Tommaso Bellini:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis. **Domenico Tortora:** Writing – review & editing, Validation, Investigation, Data curation. **Giacomo Brisca:** Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Marcello Mariani:** Writing – review & editing, Investigation, Formal analysis, Data curation.

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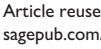

Letter to the Editor Regarding the Article: "Enterovirus and Paraechovirus Meningitis in Neonates: Which Is the Difference?"

Giacomo Brisca, MD, PhD[1], Tommaso Bellini, MD[2], and Marcello Mariani, MD[3]

Keywords
parechovirus, enterovirus, newborns, central nervous system, viral infection

Dear Editor

We really appreciate the recent manuscript authored by Picone and colleagues,[1] as it addresses a topic of great interest to us.

The authors describe a series of 18 newborns with meningitis by enterovirus (EV) and parechovirus (HpEV) with a favorable outcome.

However, we believe that 2 main points deserve discussion.

First, no patients in their series underwent magnetic resonance imaging (MRI) of the brain. In our opinion, this represents a strong limitation, as brain abnormalities are commonly reported in infants with HpEV and EV infections (36% in our experience,[2] similar to other studies).[3]

Furthermore, as the authors state, recent evidence supports a correlation between the severity of neurological outcomes in HpEV central nervous system (CNS) infections and white matter changes observed by MRI.[4]

Therefore, we suggest that all infants with EV or HpEV CNS infection be considered for an MRI, especially if a brain ultrasonography is inconclusive or equivocal or if there are symptoms suggestive of CNS involvement.

The second point concerns the possible distinguishing features between EV and HpEV infection. We agree with the authors that distinguishing between the 2 etiologies is often not clinically possible and that some subtle laboratory differences can be emphasized, such as the mild increase in C-reactive protein detected in patients with EV infection. Unfortunately, Picone et al did not report on ferritin levels in their patients. As reported also by others,[5] in our study,[2] we found a significantly increased ferritin level in neonates with HpEV infection. In our opinion, hyper-ferritinemia may represent a specific diagnostic clue for HpEV infection and should be included in the diagnostic approach to febrile infants. In addition, ferritin monitoring may allow early interception of the most fearful complication of HpEV infection, namely hemophagocytic lymphohistiocytosis, and initiation of appropriate immunomodulatory treatment. Further research will help us confirm our observations.

Author Contributions

GB: Writing – review & editing, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.
TB: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization.
MM: Writing – review & editing, Investigation, Formal analysis, Data curation, Methodology, Conceptualization.

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Clinical Features, Bacterial Coinfection Risk, and Management of Influenza in Febrile Infants

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Abstract

Objectives. Influenza in febrile infants ≤90 days represents a diagnostic and management challenge. This study aimed to characterize the clinical presentation, inflammatory profile, risk of bacterial coinfection, and impact of rapid antigen tests in this population across eight consecutive epidemic seasons. **Methods.** Single-centre retrospective study conducted at a tertiary pediatric emergency department in Genoa, Italy. Infants ≤90 days with laboratory-confirmed influenza were enrolled. Clinical, laboratory and management variables were analyzed across three age subgroups (≤28, 29–60, 61–90 days) and across pre- and post-antigen test introduction periods. **Results.** Seventy-seven infants were included (median age 52 days; 81.8% influenza A). Fever was universal. The most common additional findings were upper respiratory symptoms (35%) and poor feeding (30%). Inflammatory markers were globally low, with median CRP and PCT levels of 0.00 mg/L and 0.13 ng/mL, respectively. Complicated influenza occurred in five cases (6.5%), all with elevated CRP and PCT levels. PCT >1.71 ng/mL was 100% specific for bacterial coinfection and pneumonia. No bacteremia or meningitis was observed. The introduction of antigenic test was associated with a significant reduction in admission rates (87.9% vs. 47.7%, p=0.001) without altering the length of stay or diagnostic workup intensity. Oseltamivir was most frequently prescribed in neonates and infants with poor feeding. **Conclusion.** Influenza in infants ≤90 days old follows a mild and self-limiting course in the majority of cases. PCT-guided decision-making and systematic antigenic test use support a tailored, risk-stratified management approach, reserving the full sepsis workup for neonates and clinically unwell infants.

Keywords: Febrile infant; Influenza; Pediatric emergency Department; Oseltamivir; Procalcitonin; Rapid diagnostic testing

1. INTRODUCTION

Fever in infants under 90 days is a challenging scenario in pediatric emergency medicine because of the need to promptly identify serious bacterial infections (SBIs) [1-4]. The prevalence of SBIs in infants ≤90 days is 5% to 20%, with urinary tract infection (UTI) being the most common [4-6]. UTIs may account for over 90% of SBIs, whereas bacteremia and meningitis are less frequent [5]. Despite extensive research over several decades, differentiating between viral and bacterial causes remains difficult, especially during the early stages of illness, when clinical manifestations are nonspecific [1-4]. Furthermore, risk-stratification algorithms are primarily designed to rule out SBIs rather than to define the cause of fever, which is viral in approximately 85% of cases in this age group [1-3,7].

Seasonal influenza burdens pediatric emergency departments (PEDs) worldwide, comprising a significant number of fever-related visits, hospitalizations, and antibiotic prescriptions among young children [4,7-9]. Influenza peaks in winter, causing up to 12% of febrile episodes in infants under 3 months [7-9]. Although typically self-limiting, some patients experience bacterialcoinfectionss or pulmonary complications, leading to longer hospital stays and increased morbidity [8,10]. Thus, infants ≤90 days are often hospitalized and undergo extensive evaluations due to concerns regarding underlying SBI [1-3,7]. Confirmed influenza infections in febrile infants reduce the risk of invasive bacterial infections (IBI), specifically bacteremia and meningitis, compared to virus-negative cases [11-13]. However, data on the risk of SBI in influenza-positive infants in this age group are still limited [6,11].

Inflammatory biomarkers, including C-reactive protein (CRP) and procalcitonin (PCT), are extensively used to support the differential diagnosis [2-4,10]. However, their efficacy in pediatric influenza cases has not been well characterized. Furthermore, their ability to discriminate in very young infants is limited because of attenuated or delayed inflammatory

responses in the early phase of infection [2-4,10,14]. In this context, rapid antigen detection tests (RADTs) for influenza, which are increasingly used in emergency settings, aid in etiological diagnosis. When positive, these tests significantly reduce the need for additional testing, antibiotic use, and hospitalization, thereby optimizing the clinical management of febrile infants [4,13,15].

Thus, this study was designed to address these gaps by offering a comprehensive characterization of influenza infection in febrile infants ≤90 days old, focusing on their clinical and laboratory phenotypes, the influence of bacterial coinfection or complications on inflammatory biomarkers and their diagnostic efficacy, and the impact of RADTs on admission rates and diagnostic workup.

2. MATERIALS AND METHODS

We conducted a single-center retrospective observational study at the PED of IRCCS Giannina Gaslini, a tertiary-level pediatric hospital in Genoa, Italy, which manages approximately 38,000 PED admissions annually. Patient enrollment occurred over eight consecutive influenza seasons, defined as the period from November 1 to March 31 of the subsequent year, spanning from the 2018–2019 season through the 2025–2026 season. This timeframe aligns with the seasonal influenza surveillance framework of the Italian RespiVirNet system and the European Centre for Disease Prevention and Control [16]. All children aged 0–90 days with confirmed influenza were included. Influenza was confirmed using nasopharyngeal multiplex real-time polymerase chain reaction (RT-PCR) or rapid antigen detection tests (RADTs). Patients were categorized into two distinct periods: the pre-RADT period, which included the initial four epidemic seasons (November 2018 – March 2023), and the post-RADT period, which encompassed the subsequent four seasons (November 2023 – March

2026). This classification corresponds to the implementation of rapid influenza antigen testing in our PED.

Patients were identified through a retrospective review of their electronic medical records. The extracted variables included age in days, sex, comorbidities, symptoms, hospital admission, length of stay (LOS), inflammatory markers, urine, blood, and cerebrospinal fluid (CSF) culture results, time from fever onset to blood sampling in hours, and oseltamivir prescription. In addition to their continuous distributions, CRP and PCT values were analyzed against the threshold levels recommended by current risk-stratification algorithms and clinical practice guidelines [1-3].

Age subgroups were categorized as neonates (≤28 days), infants (29–60 days), and infants (61–90 days old). Complicated influenza was characterized by laboratory-confirmed bacterialcoinfectionn, evidenced by a positive culture from blood, urine, or CSF, or radiologically confirmed pneumonia. A positive urine culture was defined as the growth of a single pathogen in the urine with colony counts of ≥100,000 CFUs/mL. Viral coinfectionn, defined as the concurrent detection of a second respiratory virus, was analyzed independently.

2.1 Statistical Analysis

Categorical variables are presented as absolute frequencies and percentages, and continuous variables as medians and interquartile ranges (IQR). Categorical comparisons used chi-square with Yates' correction or Fisher's exact test for sparse cells. Odds ratios and confidence intervals were calculated only when cell counts were sufficient to yield stable estimates. Otherwise, group differences are reported as proportions with Fisher's exact test p-values. Continuous variables were compared using the Mann–Whitney U test. Statistical significance

was set at $p<0.05$. Missing laboratory values were handled by complete-case analysis; no imputation was performed. All statistical analyses were performed using GraphPad Prism version 9.1.0 for Windows (GraphPad Software, San Diego, California, USA, www.graphpad.com).

2.2 *Ethical Considerations*

The study was conducted in accordance with the Declaration of Helsinki. Patient data were handled per applicable data protection regulations. Given the retrospective observational design with no modification to clinical care, a formal ethics committee waiver was applied. This study was conducted and reported in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines for cohort and cross-sectional studies.

3. RESULTS

Seventy-seven infants aged ≤90 days with confirmed influenza were included. Demographic and clinical characteristics are presented in Table 1. The median age was 52 days (IQR 37–69). Neonates aged ≤28 days were 15.6% (n=12), those aged 29–60 days were 44.2% (n=34), and infants aged 61–90 days were 40.3% (n=31). Males comprised 64.9% of the sample population. Influenza A was responsible for 81.8% of the cases. Figure 1 shows the influenza incidence per 10,000 pediatric emergency department (PED) admissions and per 100 febrile infants.

Fever was observed in all patients (100%). Additional clinical findings included upper respiratory tract (URT) symptoms such as cough or rhinitis (35%), poor feeding (30%), somnolence (10%), dyspnea (6.5%), and cutaneous rash (2.5%). Notably, no gastrointestinal

145 symptoms were observed. Figure 2 illustrates the distribution and overlap of the symptoms
146 and clinical features at admission.
147 Blood tests were performed in 65 patients (84.4%). The median time from fever onset to
148 blood sampling was 8 hours (IQR 4–24). A total of 50 infants (64.9%) were hospitalized, with a
149 median LOS of 3 days (IQR 3–5). None of the patients required intensive care. Oseltamivir
150 was given in 8 cases (10.4%), all hospitalized. Urinalysis was performed in all patients.
151 Table 2 compares the three age groups. Hospital admission rates significantly decreased with
152 age (p=0.002). The LOS was 3 days for all groups (p=0.851). Oseltamivir was most prescribed
153 to neonates aged ≤28 days, though not statistically significant. No significant differences
154 were found in CRP, PCT, neutrophil, lymphocyte, and monocyte levels across the age groups.
155 Platelet counts increased with age (p=0.002), corresponding to a longer interval from fever
156 onset to bloodwork in older infants (p=0.002). WBC counts were highest in neonates
157 (p=0.040). The rates of complicated influenza were similar across age groups.
158 Table 3 compares the pre- (n=33) and post-RADT (n=44) periods. The introduction of
159 influenza RADT significantly reduced admission rates from 87.9% to 47.7% (p=0.001). Blood
160 test use did not significantly decrease (p=0.093), whereas oseltamivir prescriptions were
161 higher post-RADT. LOS remained three days for both periods. Median CRP levels were higher
162 post-RADT (p=0.020), possibly due to the longer time from fever onset to blood sampling
163 (median 10 vs. 8 hours, p=0.174).
164 Among the cohort, five infants (6.5%) met the criteria for complicated influenza. Three
165 patients had concurrent UTIs, all caused by *Escherichia coli*, while two had radiologically
166 confirmed pneumonia. Blood cultures were performed in 38 patients (49.5%). One culture
167 positive for *Staphylococcus epidermidis* was deemed a contaminant and excluded. Three
168 lumbar punctures were performed, all of which yielded negative results. Four infants had

viracoinfectionsns. Patients with complicated cases showed significantly higher CRP and PCT levels ($p<0.001$ and $p=0.001$, respectively). A CRP level >2 mg/L was present in 80.0% of complicated cases vs. 6.8% in non-complicated cases ($p=0.001$), while a PCT level >0.5 ng/mL was present in 60.0% vs. 5.1% ($p=0.004$), and a PCT level >1.71 ng/mL was exclusive to complicated cases (60.0% vs. 0.0%, $p<0.001$). Complicated influenza was linked to a longer LOS (median 7 vs. 3 days, $p=0.011$). Infants with CRP >2 mg/L were sampled later than those with normal CRP (median 21 vs. 6 hours from fever onset, $p=0.029$). Those with PCT >0.5 ng/mL had an even longer interval (median 24 vs. 6 hours, $p=0.021$). Given the small number of complicated cases (n=5), odds ratios were not estimated. The main results are presented in Table 4.

Oseltamivir was given to 8 infants (10.4%), all hospitalized. The highest prescription rate was observed in neonates ≤28 days (25.0%). Poor feeding was the only symptom significantly associated with oseltamivir prescription ($p=0.010$).

4. DISCUSSION

This study examined the clinical presentation, inflammatory profile, and management of influenza in infants aged ≤90 days evaluated at a tertiary PED over eight influenza seasons. This study contributes to the limited evidence available for this age group.

During seasonal influenza epidemics, infants in the first months of life are a vulnerable population, with influenza causing many febrile illnesses [7,9]. Furthermore, these patients are too young to be vaccinated and rely on indirect protection, such as immunizing caregivers, family members, and pregnant women [17-23]. Maternal influenza immunization reduces confirmed influenza illness by over 60% in infants up to six months [19-20,23]. However, evidence suggests that influenza in this age group often leads to mild disease and

193 favorable outcomes, with limited hospitalization and intensive care needs [7,15,21]. Our
194 findings are consistent with these results.
195 Our subgroup analysis showed increased admission rates and time to blood sampling in
196 neonates, consistent with conservative protocols for this age group. Neonates ≤28 days often
197 undergo full sepsis evaluation and may receive antibiotics regardless of symptoms, while
198 infants 29–90 days are managed with selective algorithms [1,6-7]. However, LOS was similar
199 across ages, indicating comparable clinical severity upon admission.
200 The incidence of bacterial infections is reduced in febrile infants aged 61–90 days who test
201 positive for respiratory viruses. However, febrile infants aged ≤60 days with viral infections
202 still have a lower, yet non-negligible risk of concomitant SBIs [12-13]. Specifically, among
203 influenza-positive neonates, the prevalence of IBIs is below 1%, with no reported cases of
204 bacterial meningitis [6-7,11]. Generally, young infants with influenza exhibit favorable
205 outcomes, including a low risk of concurrent SBIs, although UTIs remain prevalent. Thus, viral
206 positivity alone cannot rule out bacterial coinfection [6-7,11,24]. The prevalence of
207 complicated influenza in our cohort (6.5%) aligns with published series, reporting SBIs
208 prevalence from 2.3% to 11% [6-7,11,15]. We observed no cases of bacteremia or meningitis,
209 reinforcing that influenza significantly reduces the likelihood of IBIs in young infants [6,11].
210 Concurrently, we identified a low but notable prevalence of UTIs (3.9%), indicating that
211 urinalysis should always be conducted [11]. In this context, inflammatory biomarkers such as
212 CRP and PCT aid in the differential diagnosis. However, their diagnostic efficacy in very young
213 infants remains suboptimal, especially in the early stages of the disease, where inflammatory
214 responses may be attenuated or delayed [1,14].
215 Our data indicate that influenza infection in early infancy is linked to a low inflammatory
216 phenotype. Most infants showed low CRP and PCT values, with normal or reduced WBC

counts, as supported by previous studies [6]. The timing of sample collection directly impacts this age group. The age-related increase in platelet counts likely reflects a longer fever-to-bloodwork interval in older infants. Reactive thrombocytosis has been noted in influenza-confirmed pediatric cohorts and is inversely correlated with age [25-26]. Conversely, elevated WBC in neonates aligns with the physiologically high leukocyte reference ranges in the first weeks of life and should not be considered as indicating a greater inflammatory burden [7-8]. Neonates were brought to the PED and sampled early (median of 4 hours from fever onset), when the acute phase response is least developed, making their biomarker values prone to underestimation [1,14]. Infants with CRP levels >2 mg/L were sampled significantly later than those with normal levels (21 versus 6 hours from fever onset, p=0.029). A similar trend was observed for those with PCT levels >0.5 ng/mL, with an even longer interval (24 versus 6 hours, p=0.021). Thus, normal CRP or PCT levels in neonates sampled early should not exclude the possibility of evolving bacterial superinfection. Therefore, a comprehensive evaluation of sepsis is essential in this age group. Notably, PCT is the most informative biomarker, serving as a more specific indicator of bacterial involvement than CRP in viral infections [8,10]. A PCT >1.71 ng/mL was 100% specific for complicated influenza [2]. However, the broad IQR of PCT in complicated cases (0.80–11.70) indicates substantial heterogeneity, necessitating a cautious interpretation.

Several studies have shown that early bedside viral identification reduces the need for ancillary testing, antibiotic prescriptions, and hospitalizations [4]. However, the effect of universal RADT adoption on admission rates and clinical practices in febrile infants has not been systematically examined. Our 2023–24 influenza incidence increase may partly reflect the introduction of RADTs in our PED, although a true epidemiological rise due to post-pandemic immune debt should not be excluded. The post-RADTs admission rate drop was

241 the most significant management change in this cohort, aligning with evidence from older
242 infants and children that bedside viral identification reduces hospitalizations [4,15]. Thus,
243 routine RADTs in algorithms for febrile infants aged 29-90 days may quickly identify influenza,
244 reducing the need for extensive and invasive diagnostics [4,6,15].
245 Oseltamivir is approved for infants aged ≥2 weeks, and the Centers for Disease Control and
246 Prevention recommends it for high-risk children under 2 with confirmed or suspected
247 influenza [27-31]. It is safe and reduces illness duration and LOS [28-30,32]. In our cohort, its
248 association with poor feeding and younger age suggests that clinicians should prioritize
249 antiviral treatment for infants perceived as the most unwell or with complications, aligning
250 with guidelines for the youngest, most vulnerable infants [21,27-32]. No patient experienced
251 any side effects. However, the low prescription rate (10.4%) despite exclusively vulnerable
252 infants indicates a gap between guidelines and real-world practice.
253 This study has some limitations. First, the retrospective single-center design may introduce
254 selection bias, which may not reflect community-level epidemiology, and information bias
255 arising from variability in clinical documentation and missing data. Second, data collection
256 was limited to the epidemic period, excluding influenza cases outside this period. Third, the
257 small number of bacterial coinfections prevented multivariate modelling and limited the
258 statistical evaluation of complicated influenza across age groups. Finally, insufficient
259 documentation of clinical appearance at presentation precluded a systematic assessment of
260 well- versus ill-appearing status, potentially biasing risk stratification.
261 In conclusion, this study examined the characteristics of influenza in infants aged ≤90 days at
262 a tertiary PED over eight epidemic seasons. In most cases, influenza presents as a mild and
263 self-limiting condition. The introduction of RADT was associated with a significant reduction
264 in admission rates without affecting the intensity of the diagnostic workup, incidence of

missed IBIs, or LOS. This supports a less invasive and more personalized management approach for low-risk patients. PCT and CRP effectively differentiated between complicated and uncomplicated influenza and identified clinically unwell infants, with a PCT threshold of >1.71 ng/mL being highly specific for bacterial superinfection or pneumonia. However, early blood sampling within the first 6–8 hours of fever onset may underestimate the inflammatory response and should not be solely relied upon to exclude evolving bacterial superinfection. Urinalysis should be systematically conducted because of the non-negligible prevalence of UTIs, even in influenza-positive infants. Importantly, the risk of IBI in neonates remains significant, and a full sepsis workup should not be deferred based solely on viral positivity in this age group. Oseltamivir was most frequently prescribed to neonates and infants with poor feeding, aligning with guidelines that prioritize antiviral treatment for the most vulnerable. Confirmation of these findings in a multicenter study would support a more tailored, risk-stratified approach, reserving aggressive workup for neonates and clinically unwell infants, while permitting conservative management in older, well-appearing influenza-positive patients.

List of Abbreviations

CRP	C-Reactive Protein
CSF	CerebroSpinal Fluid
IBI	Invasive Bacterial Infection
IQR	InterQuartile Range
LOS	Length of Stay
OR	Odds Ratio
PCT	Procalcitonin
PED	Pediatric Emergency Department
RADT	Rapid Antigen Detection Test
RT-PCR	Real Time Polymerase Chain Reaction
SBI	Serious Bacterial Infection
UTI	Urinary Tract Infection
WBC	White Blood Cell

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Table 1. Demographic, clinical and laboratory characteristics of the study population. Continuous variables are reported as median (IQR); categorical variables as n (%). Missing data are reported for each laboratory variable. †Among admitted patients only (n=50). WBC, white blood cell count; CRP, C-reactive protein; PCT, procalcitonin; IQR, interquartile range.

Characteristic	Value	Missing, n
Total patients, n	77	—
Age, days, median (IQR)	52 (37–69)	0
≤28 days (neonates), n (%)	12 (15.6%)	0
29–60 days, n (%)	34 (44.2%)	0
61–90 days, n (%)	31 (40.3%)	0
Male sex, n (%)	50 (64.9%)	0
Influenza A, n (%)	63 (81.8%)	0
Influenza B, n (%)	14 (18.2%)	0
Hospital admission, n (%)	50 (64.9%)	0
Length of stay, days, median (IQR)†	3 (3–5)	—
Blood tests performed, n (%)	65 (84.4%)	12
WBC, cells/µL, median (IQR)	7030 (5660–8200)	12
Neutrophils, cells/µL, median (IQR)	2840 (1930–3890)	12
Lymphocytes, cells/µL, median (IQR)	2500 (1850–3780)	12
Monocytes, cells/µL, median (IQR)	860 (570–1200)	12
CRP, mg/L, median (IQR)	0.00 (0.00–0.67)	12
PCT, ng/mL, median (IQR)	0.13 (0.10–0.19)	13
Platelets, cells*10^3/µL, median (IQR)	362 (280–441)	12
Fever-to-bloodwork time in h, median (IQR)	8 (4–24)	12
Oseltamivir prescribed, n (%)	8 (10.4%)	0
Viral co-infection (virus–virus), n (%)	4 (5.2%)	0
Bacterial co-infection (virus–bacteria), n (%)	3 (3.9%)	0
Complications (pneumonia), n (%)	2 (2.6%)	0

Table 2. Clinical and laboratory characteristics according to age subgroups. Continuous variables are reported as median (IQR) and categorical variables as n (%). † Only among admitted patients. WBC, white blood cell count; CRP, C-reactive protein; PCT, procalcitonin; LOS, length of stay; IQR, interquartile range.

	≤28 days (n=12)	29–60 days (n=34)	61–90 days (n=31)	*p*
Hospital admission, n (%)	11 (91.7%)	26 (76.5%)	13 (41.9%)	**0.002**
Male sex, n (%)	6 (50.0%)	24 (70.6%)	20 (64.5%)	0.369
Comorbidities, n (%)	1 (8.3%)	7 (20.6%)	1 (3.2%)	0.094
Oseltamivir, n (%)	3 (25.0%)	3 (8.8%)	2 (6.5%)	0.187
Complicated influenza, n (%)	1 (8.3%)	1 (2.9%)	3 (9.7%)	0.524
Blood tests performed, n (%)	11 (91.7%)	30 (88.2%)	24 (77.4%)	0.366
WBC, cells/µL, median (IQR)	8200 (6350–8995)	6150 (5028–7335)	7715 (5818–8735)	**0.040**
Neutrophils, cells/µL, median (IQR)	2670 (2100–4140)	2730 (1420–3612)	3230 (2112–4395)	0.358
Lymphocytes, cells/µL, median (IQR)	2500 (1830–4395)	2235 (1680–3735)	2850 (2262–3700)	0.450
Monocytes, cells/µL, median (IQR)	1140 (875–1405)	875 (580–1148)	700 (560–1012)	0.082
CRP, mg/L, median (IQR)	0.00 (0.00–0.00)	0.00 (0.00–0.56)	0.59 (0.00–1.74)	0.065
PCT, ng/mL, median (IQR)	0.11 (0.08–0.13)	0.14 (0.11–0.18)	0.13 (0.10–0.26)	0.186
Platelets, cells*10^3/µL, median (IQR)	265 (220–314)	361 (294–434)	435 (310–468)	**0.002**
Fever-to-bloodwork time in h, median (IQR)	4 (3–15)	6 (3–12)	12 (8–24)	**0.002**
LOS, days, median (IQR)†	3	3	3	0.851

Table 3. Clinical management and laboratory parameters before and after the introduction of universal rapid antigen diagnostic testing (October 2023). Continuous variables are reported as median (IQR) and categorical variables as n (%). † Only among admitted patients. IQR, interquartile range.

Variable	Pre-Oct 2023 (n=33)	Post-Oct 2023 (n=44)	*p*
Hospital admission, n (%)	29 (87.9%)	21 (47.7%)	**0.001**
Blood tests performed, n (%)	31 (93.9%)	34 (77.3%)	0.093
Oseltamivir prescribed, n (%)	1 (3.0%)	7 (15.9%)	0.146
Length of stay, days, median (IQR)†	3 (3–5)	3 (2–4)	0.226
Fever-to-bloodwork time in h, median (IQR)	8 (2.5–12)	10 (4–24)	0.174

Table 4. Inflammatory biomarkers and clinical outcomes in complicated versus uncomplicated influenza. Continuous variables are reported as median (IQR); categorical variables as n (%).*Complicated infcoinfectionfined as confirmed bacterial co-infecticcoinfectionulture from blood, urine, or CSF) or radiologically confirmed pneumonia; cases with viral co-infection were considered uncomplicated. †Among admitted patients only. CRP, C-reactive protein; PCT, procalcitonin; IQR, interquartile range; CSF, cerebrospinal fluid; LOS, length of stay.

Variable	Complicated* (n=5)	Uncomplicated (n=72)	*p*
CRP, mg/L, median (IQR)	7.09 (3.30–8.94)	0.00 (0.00–0.64)	**<0.001**
PCT, ng/mL, median (IQR)	6.55 (0.80–11.70)	0.12 (0.10–0.18)	**0.001**
CRP >2 mg/L, n (%)	4 (80.0%)	5 (6.8%)	**0.001**
PCT >0.5 ng/mL, n (%)	3 (60.0%)	4 (5.1%)	**0.004**
PCT >1.71 ng/mL, n (%)	3 (60.0%)	0 (0.0%)	**<0.001**
Neutrophils >4090 cells/µL, n (%)	2 (40.0%)	12 (16.9%)	0.233
Length of stay, days, median†	7	3	**0.011**

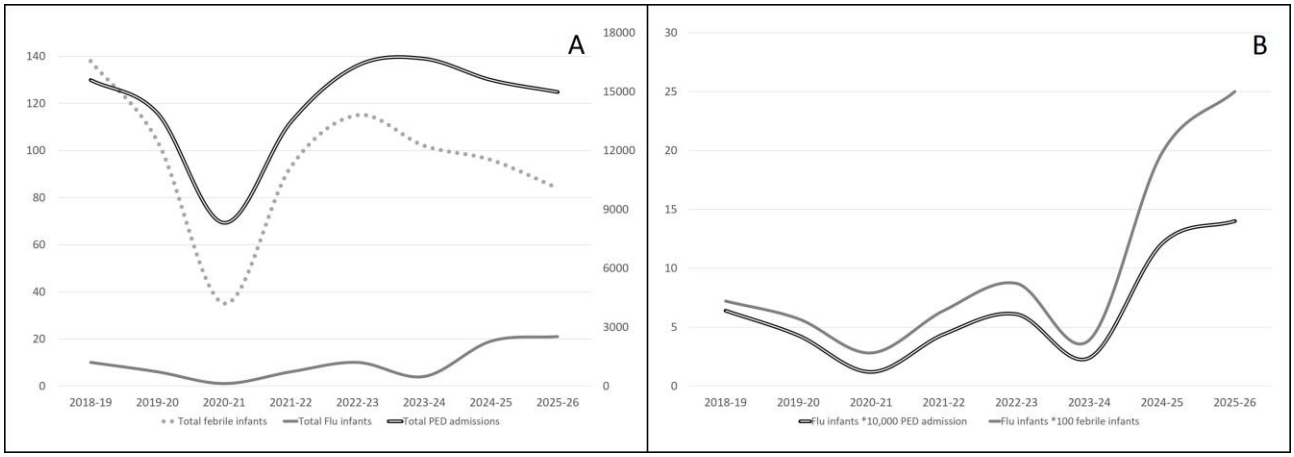

Figure 1 legend. Influenza incidence among infants ≤90 days of age across eight consecutive epidemic seasons (2018–2026). Panel A shows the absolute annual counts of influenza-positive infants, total febrile infants, and total PED admissions (left y-axis: case counts; right y-axis: total PED admissions). Panel B shows the normalized incidence rates expressed as influenza-positive infants per 10,000 total PED admissions and per 100 febrile infants. The marked reduction observed during the 2020–21 season reflects the suppression of influenza circulation during the COVID-19 pandemic. The progressive rise in both incidence indicators from the 2023–24 season onward coincided with the introduction of rapid antigen detection testing in our department.

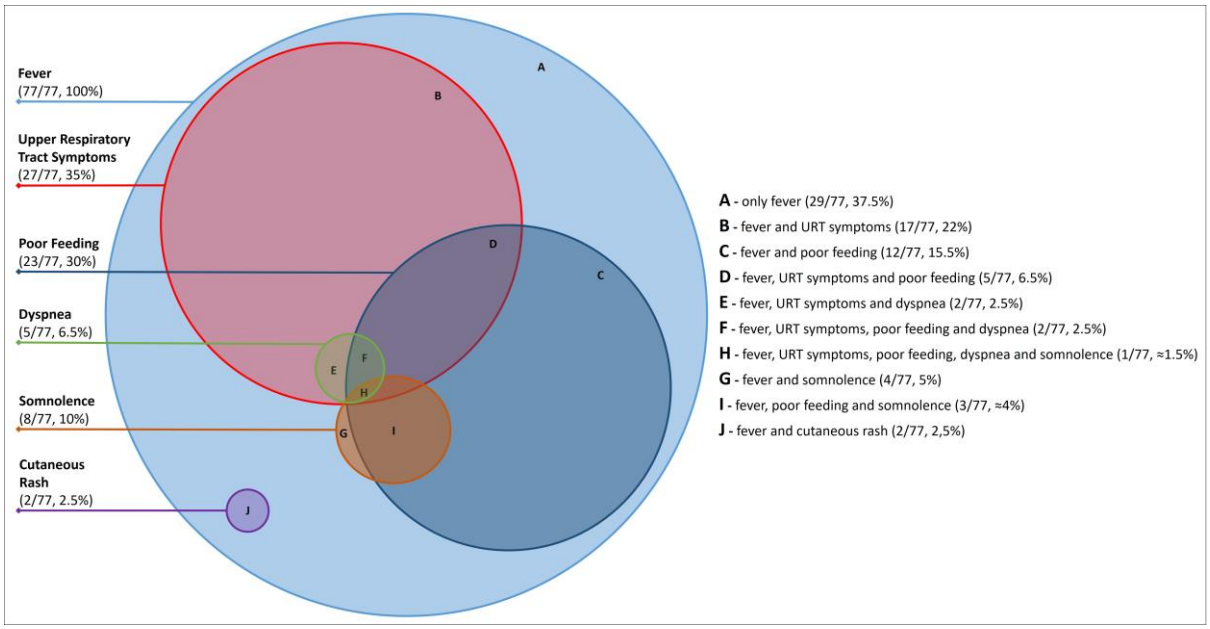

Figure 2 legend. Distribution and overlap of presenting symptoms among 77 influenza-positive infants ≤90 days of age. The diagram illustrates the frequency of individual symptoms and their co-occurrence. Regions A–J represent distinct symptomatic combinations: fever alone was the most frequent presentation (A; 37.5%), followed by fever with URT symptoms (B; 22%) and fever with poor feeding (C; 15.5%). More complex symptom clusters involving three or more features were observed in a minority of patients (D–H; 2.5–6.5%). Cutaneous rash occurred exclusively without other associated symptoms beyond fever (J; 2.5%).

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Clinical Course, Laboratory Findings, and Prognosis of SARS-CoV-2 Infection in Infants up to 90 Days of Age: A Single-Center Experience and a Proposal for a Management Pathway

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Abstract: Aim: To provide a comprehensive description of the clinical features, biochemical characteristics, and outcomes of infants up to 90 days old with COVID-19. Moreover, to assess the severity of the disease and propose an effective management pathway. Methods: Retrospective single-center study spanning three years. Patient data includes age, sex, symptoms, comorbidities, blood and urine test results, cultures, admission, length of stay, therapies, intensive care unit admission, and mortality. Results: A total of 274 patients were enrolled in the study, comprising 55% males. Among them, 60 patients (22%) were under the age of 29 days, while 214 (78%) fell within the 29 to 90 days age range. The overall incidence of SARS-CoV-2 infections was 0.28 per 10,000 Pediatric Emergency Department admissions. Blood inflammatory markers showed no significant abnormalities, and there were no recorded instances of positive blood cultures. Less than 1% of infants showed urinary tract infections with positive urine cultures, and 1.5% of patients had a concurrent RSV infection. Hospitalization rates were 83% for neonates and 67% for infants, with a median length of stay (LOS) of 48 h for both age groups. None of the patients required admission to the Pediatric or Neonatal Intensive Care Unit, and only one required High Flow Nasal Cannula (HFNC). No secondary serious bacterial infections were observed, and all hospitalized patients were discharged without short-term sequelae. No deaths were reported. Discussion and Conclusions: Infants with COVID-19 generally exhibit milder or asymptomatic forms of the disease, making home management a viable option in most cases. Blood tests, indicative of a mild inflammatory response, are recommended primarily for children showing symptoms of illness. Hospitalization precautions for infants without apparent illness or comorbidities are deemed unnecessary. Given the evolving nature of experiences with COVID-19 in infants, maintaining a high level of clinical suspicion remains imperative.

Keywords: clinical severity; COVID-19 epidemiology; febrile infant; pediatric emergency department; prognosis

1. Introduction

Coronavirus Disease-19 (COVID-19) is caused by Severe Acute Respiratory Syndrome-Coronavirus-2 (SARS-CoV-2) [1]. However, information about pediatric patients, especially those under 90 days of age, remains fragmented, scarce, and often contradictory [2–4]. Knowledge of these young patients has been extrapolated from older pediatric cases, with a limited number of research and case series publications during the ongoing pandemic [2].

The potential risk factors for community-acquired COVID-19 in very young individuals are unclear, with speculation about whether immature immune systems pose a risk or play a protective role [2,5–8]. Several risk stratification scores for COVID-19 in young infants have been proposed but often show conflicting results, and the percentage of infants experiencing severe COVID-19 varies widely across different studies [1,3,9].

In infants affected by COVID-19, fever emerges as the most common symptom and is one of the most frequent reasons for consultation in the pediatric emergency department (PED) [2,10].

Fever without a clear source may be the sole presenting symptom, and in patients aged 90 days or younger, physical examination and laboratory tests may not be consistently differentiate between viral infections, including SARS-CoV-2, and Severe Bacterial Infection (SBI) [11].

Several algorithms have been proposed to identify subgroups at higher risk of SBI, constituting up to 12% of febrile children aged 90 days or younger [11–15]. Among these, the American Academy of Pediatrics guidelines and the "Step-by-Step" approach are the most commonly used, demonstrating a lower incidence of undiagnosed SBI, compared to other scoring systems [13,14]. However, these approaches have been reported to be less sensitive in children with a short duration of fever, often leading to hospitalization and a comprehensive sepsis workup [10,11,14,16]. Moreover, these algorithms do not include the use of rapid diagnostic tests for viral infections, including COVID-19 [11,13,14].

The primary aim of our study was to describe the clinical and biochemical characteristics and outcomes of neonates and young infants with COVID-19 to evaluate disease severity and the need for diagnostic investigations and hospitalization.

Simultaneously, we sought to propose a management pathway for neonates and young infants with COVID-19 in PEDs.

2. Materials and Methods

We conducted a retrospective analysis by collecting data from the medical records of infants admitted to the PED of a tertiary children's hospital (IRCCS Istituto Giannina Gaslini, Genoa, Italy) spanning from 1 November 2020 to 31 October 2023.

We included all patients aged $\leq$90 days with a confirmed positive antigenic nasal swab for SARS-CoV-2, or detection of SARS-CoV-2 RNA using a real-time quantitative reverse transcriptase polymerase chain reaction (RTq-PCR) from either a nasal swab or saliva sample.

Patients accessing our PED before 1 November 2020 were excluded, as comprehensive screening for SARS-CoV-2 had not yet been implemented for all admitted patients during that period.

Demographic data such as age, sex, presence of older siblings, comorbidities, and perinatal history, along with symptoms on admission, administered therapies, and results of blood and urine tests were collected. Outcome data, length of hospitalization, admission to the neonatal (NICU) or pediatric intensive care unit (PICU), need for invasive or non-invasive ventilation, occurrence of complications, and mortality were also documented.

Patients were categorized into two groups based on age: those younger than 28 days (referred to as the "neonates group") and those 28 days or older (referred to as the "infants group"). Subsequently, patients were further stratified into febrile and afebrile groups. Among febrile patients, a subdivision was made based on the time elapsed between the onset of fever and admission to the PED, using six hours of fever as the cut-off. A flowchart of the inclusion and exclusion criteria is shown in Figure 1.

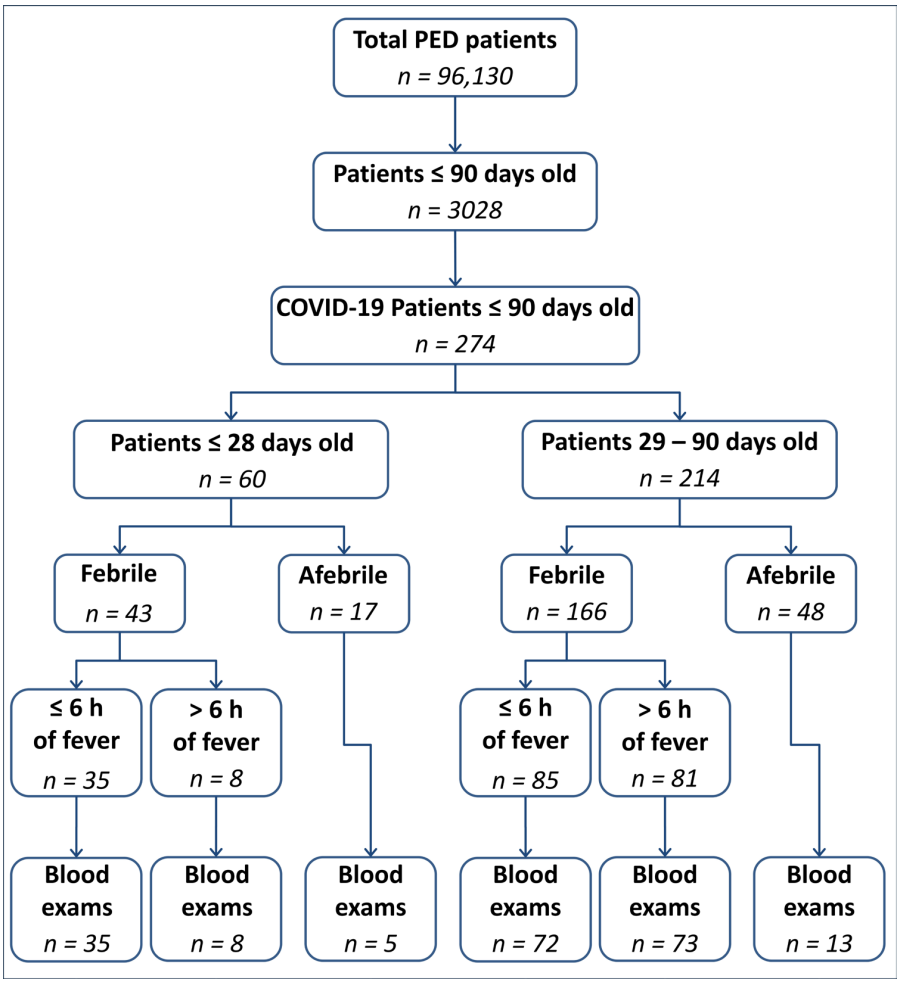

Figure 1. Flowchart of the inclusion and exclusion criteria. PED = Pediatric Emergency Department; COVID-19 = CoronaVirus Disease 19.

The study did not seek local ethics committee approval, as it falls within the purview of the institute's ethics committee, which had previously approved COVID-19-related data collection (Regione Liguria Ethical Board; IRB#370/2020).

Statistical Analysis

Mean and standard deviation (SD) were utilized for normally distributed variables, while median and interquartile range (IQR) were represented for non-normally distributed variables. Categorical variables were expressed as numbers and percentages. Group differences were assessed using Kruskal–Wallis test or Mann–Whitney U-test for continuous variables, and chi-square or Fisher's exact test for categorical variables. Statistical significance was established at $p < 0.05$, and all values were determined using two-tailed tests. Multivariable logistic or linear regression analysis, based on variable types, was performed to identify clinical and laboratory differences between non-hospitalized and hospitalized patients, as well as factors influencing the length of stay. Only variables that demonstrated statistical significance at the univariable level were considered for multivariable models. All statistical analyses were conducted using IBM SPSS Statistics for Windows Version 21.0 (IBM Corp., Armonk, NY, USA).

3. Results

Demographic and Clinical Findings

A total of 274 outpatients were included in the study, reflecting overall incidence of 0.28 SARS-CoV-2 infections per 10,000 PED admissions. The median age was 47 days (IQR

31–68), with 45% being female. SARS-CoV-2 infection was diagnosed in 60 neonates and 214 infants, and no season distribution was observed SARS-CoV-2 infection was detected in 9% of patients aged ≤90 days. The demographic and clinical data are summarized in Table 1. A total of 43.5% patients had at least one older sibling with equal distribution between the two age groups (46.5% of neonates and 42% of infants). However, data were missing for 99 patients due to incomplete medical history.

Table 1. Demographic, clinical, and prognostic findings of enrolled patients. IQR = interquartile range; LOS = length of stay; RSV = respiratory syncytial virus; GI = gastro-intestinal; LRT = lower respiratory tract; URT = upper respiratory tract; HFNC = high flow nasal cannula.

	Total ***n*** **= 274**	**Patients <29 Days Old** ***n*** **= 60**	**Patients ≥29 Day Old** ***n*** **= 214**	***p***
Age, median (IQR 25–75)	47 (31–68)	16 (10.75–22.25)	54.5 (41–71)	<0.0001
Sex, male (%)	152 (55.0%)	33 (55%)	119 (55.5%)	0.93
Comorbidities, yes (%)	23 (9%)	5 (8.5%)	18 (9%)	0.81
Older sibling, yes (%)	76 (43.5%)	22 (47%)	54 (42%)	0.58
Maternal GBS, positive (%)	17 (6.5%)	6 (10.5%)	11 (5.5%)	0.19
Admission, yes (%)	195 (71%)	50 (83.5%)	145 (67.5%)	0.018
LOS in h, median (IQR 25–75)	48 (48–72)	48 (48–78)	48 (48–72)	0.94
Poor clinical condition, yes (%)	8 (3%)	3 (5%)	5 (2.5%)	0.27
Asymptomatic, yes (%)	19 (7%)	6 (10%)	13 (6%)	0.29
Fever, yes (%)	209 (76%)	43 (71.5%)	166 (77.5%)	0.34
Fever < 6 h, yes (%)	120 (57.5%)	35 (81%)	85 (51%)	0.004
Blood exams, yes (%)	206 (75%)	48 (80%)	158 (74%)	0.32
Blood exams in fever, yes (%)	188 (90%)	43 (100%)	145 (87%)	0.013
Blood exams in afebrile, yes (%)	65 (24%)	5 (29.5%)	13 (27%)	0.85
Urine exams, yes (%)	192 (70%)	38 (63%)	154 (78%)	0.19
Positive nitrites, yes (%)	2 (1%)	0 (%)	2 (1.5%)	0.47
Leukocyturia, yes (%)	9 (4.5%)	0 (%)	9 (6%)	0.12
Urine culture collected, yes (%)	66 (34%)	12 (31.5%)	54 (35%)	0.68
Urine culture, positive (%)	2 (3%)	0 (0%)	2 (3.5%)	0.49
Blood culture collected, yes (%)	47 (23%)	11 (23%)	36 (22.5%)	0.98
Blood culture, positive (%)	0 (0%)	0 (%)	0 (%)	/
RSV coinfection, yes (%)	5 (2%)	0 (0%)	5 (2.5%)	0.23
GI symptoms, yes (%)	18 (6.5%)	3 (5%)	15 (7%)	0.57
Poor feeding, yes (%)	61 (22%)	19 (31.5%)	42 (19.5%)	0.047
LRT symptoms, yes (%)	19 (7%)	2 (3.5%)	17 (8%)	0.21
URT symptoms, yes (%)	93 (34%)	21 (35%)	72 (33.5%)	0.84
Dyspnea, yes (%)	16 (6%)	3 (3.5%)	13 (6%)	0.69
Apnea, yes (%)	6 (2%)	1 (1.5%)	5 (2.5%)	0.75
Cutaneous rash, yes (%)	7 (2.5%)	1 (1.5%)	6 (3%)	0.62
Oxygen supplementation, yes (%)	10 (3.5%)	3 (3.5%)	7 (3.5%)	0.52
HFNC, yes (%)	1 (0.5%)	0 (0%)	1 (0.5%)	0.59

We observed a higher hospitalization rate in the newborn group compared to the infant group (83.5% vs. 67.5%, $p = 0.018$), although there were no significant differences in the length of hospital stay (LOS).

Twenty-three children (8.5%) had comorbidities, including premature birth, with three premature patients in the newborn group and four in the infant group.

While there was a trend towards a longer length of stay for patients with comorbidities (72 h, IQR 48–96, vs. 48 h, IQR 48–72) this difference did not reach statistical significance ($p = 0.09$). However, patients with comorbidities did not show a higher hospitalization rate ($p = 0.2$).

Seventeen patients (6.2%) had a history of positive maternal vaginal swabs for Group B *Streptococcus* (GBS). In eleven cases, complete intrapartum antibiotic prophylaxis was administered, while the remaining six patients underwent blood tests at birth and in the days following birth. None of the patients presented early neonatal sepsis. Table 1 shows the main symptoms upon admission to the PED. Nineteen patients (7%) were completely asymptomatic, evenly distributed between the neonates and infants groups. Eight infants (3%) were noted to have poor clinical conditions. Fever was the main presenting symptom in 206 patients (76%), occurring as an isolated symptom in 39% of cases, with no significant difference between the two groups ($p = 0.34$). Neonates, however, showed a statistically significant increase in the prevalence of poor feeding ($p = 0.047$) and a shorter time interval between the onset of fever and PED arrival ($p = 0.004$).

Blood and urine tests were performed in 206 (75%) and 192 (70%) patients, respectively. The results are summarized in Tables 1–5.

Table 2. Comparison of inflammatory marker values between the two age groups within six hours of fever onset. Continuous variables are described as median and interquartile range (IQR), and categorical variables as absolute and relative frequencies. CRP = C-reactive protein; PCT = procalcitonin; WBC = white blood cells; ANC = absolute neutrophils count.

	Total Patients *n* = 107	**Patients <29 Days Old *n* = 35**	**Patients ≥29 Days Old *n* = 72**	***p***
CRP in mg/dL, median (IQR 25–75)	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.00 (0.00–0.08)	0.34
CRP > 2 mg/dL, yes (%)	11 (10%)	0 (0%)	11 (15%)	0.014
PCT in ng/mL, median (IQR 25–75)	0.11 (0.05–0.14)	0.11 (0.08–0.13)	0.10 (0.05–0.14)	0.85
PCT > 0.5 ng/mL, yes (%)	2 (2%)	1 (3%)	1 (1.5%)	0.59
WBC in cells/mm^3, median (IQR 25–75)	7100 (5300–8750)	7900 (6000–8850)	6650 (5150–8375)	0.16
WBC > 10,000 cells/mm^3, yes (%)	12 (11%)	3 (8.5%)	9 (12.5%)	0.54
ANC in cells/mm^3, median (IQR 25–75)	2300 (1750–3400)	2800 (2100–3600)	2150 (1500–3200)	0.03
ANC < 1000 cells/mm^3, yes (%)	3 (3%)	1 (3%)	2 (3%)	0.98
ANC > 5000 cells/mm^3, yes (%)	6 (6%)	0 (0%)	6 (8.5%)	0.07

Table 3. Comparison of inflammatory marker values between the two age groups more than six hours after fever onset. Continuous variables are described as median and interquartile range (IQR), and categorical variables as absolute and relative frequencies. CRP = C-reactive protein; PCT = procalcitonin; WBC = white blood cells; ANC = absolute neutrophils count.

	Total Patients *n* = 81	**Patients <29 Days Old *n* = 8**	**Patients ≥29 Days Old *n* = 73**	***p***
CRP in mg/dL, median (IQR 25–75)	0.00 (0.00–0.00)	0.00 (0.00–0.04)	0.00 (0.00–0.00)	0.51
CRP > 2 mg/dL, yes (%)	2 (2.5%)	1 (12.5%)	1 (1.5%)	0.054
PCT in ng/mL, median (IQR 25–75)	0.11 (0.05–0.16)	0.15 (0.10–0.19)	0.10 (0.05–0.15)	0.65
PCT > 0.5 ng/mL, yes (%)	1 (1%)	0 (0%)	1 (1.5%)	0.73
WBC in cells/mm^3, median (IQR 25–75)	7200 (5150–9800)	10,200 (7400–11,000)	6500 (4300–8900)	0.005
WBC > 10,000 cells/mm^3, yes (%)	19 (23.5%)	5 (62.5%)	14 (19%)	0.006
ANC in cells/mm^3, median (IQR 25–75)	1970 (1000–2525)	2100 (1600–2550)	1600 (1000–2500)	0.25
ANC < 1000 cells/mm^3, yes (%)	18 (22%)	2 (25%)	16 (22%)	0.84
ANC > 5000 cells/mm^3, yes (%)	5 (6%)	1 (12.5%)	4 (5.5%)	0.43

Table 4. Comparison of age, sex, and values of inflammatory markers in patients aged ≤28 days with or without fever at the time of PED admission. Continuous variables are described as median and interquartile range (IQR), and categorical variables as absolute and relative frequencies. CRP = C-reactive protein; PCT = procalcitonin; WBC = white blood cells; ANC = absolute neutrophil count.

	Total < 29 Days Old Patients *n* = 48	**Febrile Patients *n* = 43**	**Afebrile Patients *n* = 5**	***p***
Age, median (IQR 25–75)	16 (10.75–22.25)	16 (10.50–22.00)	16 (11.00–23.00)	0.67
Sex, male (%)	25 (52%)	22 (51%)	3 (60%)	0.70
CRP in mg/dL, median (IQR 25–75)	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.60
CRP > 2 mg/dL, yes (%)	1 (2%)	1 (2.5%)	0 (0%)	0.73
PCT in ng/mL, median (IQR 25–75)	0.11 (0.07–0.15)	0.12 (0.08–0.15)	0.08 (0.04–0.12)	0.87
PCT > 0.5 ng/mL, yes (%)	1 (2%)	1 (2.5%)	0 (0%)	0.73
WBC in cells/mm^3, median (IQR 25–75)	7900 (6300–9200)	7950 (6400–9975)	8500 (6750–10,400)	0.20
WBC > 10,000 cells/mm^3, yes (%)	9 (19%)	8 (18.5%)	1 (20%)	0.94
ANC in cells/mm^3, median (IQR 25–75)	2500 (1950–3500)	2450 (2000–3475)	1500 (1450–2150)	0.065
ANC < 1000 cells/mm^3, yes (%)	1 (2%)	3 (7%)	0 (0%)	0.54
ANC > 5000 cells/mm^3, yes (%)	0 (0%)	1 (2.5%)	0 (0%)	0.73

Table 5. Comparison of age, sex, and values of inflammatory markers in patients aged 29–90 days with or without fever at the time of PED admission. Continuous variables are described as median and interquartile range (IQR), and categorical variables as absolute and relative frequencies. CRP = C-reactive protein; PCT = procalcitonin; WBC = white blood cells; ANC = absolute neutrophil count.

	Total 29–90 Days Old Patients *n* = 158	**Febrile Patients *n* = 145**	**Afebrile Patients *n* = 13**	***p***
Age, median (IQR 25–75)	54.5 (41–71)	55.5 (42–72)	51.00 (37.75–67.75)	0.26
Sex, male (%)	88 (55.5%)	81 (56%)	7 (54%)	0.88
CRP in mg/dL, median (IQR 25–75)	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.27
CRP > 2 mg/dL, yes (%)	12 (7.5%)	12 (8%)	0 (0%)	0.28
PCT in ng/mL, median (IQR 25–75)	0.09 (0.03–0.14)	0.10 (0.05–0.15)	0.00 (0.00–0.05)	0.32
PCT > 0.5 ng/mL, yes (%)	2 (1.5%)	2 (1.5%)	0 (0%)	0.66
WBC in cells/mm^3, median (IQR 25–75)	6600 (4800–8900)	6600 (4800–8900)	8900 (5900–9550)	0.19
WBC > 10,000 cells/mm^3, yes (%)	25(16%)	23 (16%)	2(15%)	0.96
ANC in cells/mm^3, median (IQR 25–75)	2000 (1285–2800)	2000 (1200–2700)	1700 (1400–2500)	0.59
ANC < 1000 cells/mm^3, yes (%)	20 (12.5%)	18 (12.5%)	2 (15%)	0.75
ANC > 5000 cells/mm^3, yes (%)	10 (6%)	10 (7%)	0 (0%)	0.32

We did not find any positive blood cultures in the 47 patients tested. Nine patients had a positive urine dipstick for nitrites and/or leukocytes, while only two infants had positive urine cultures, one for *Klebsiella pneumoniae* and one for *Escherichia coli*. Five patients, all from the infant group, presented a coinfection with respiratory syncytial virus (RSV). Lumbar puncture was performed in only one newborn due to fever without a source and poor clinical conditions.

All 18 patients with gastrointestinal symptoms tested negative for bacterial cultures and viral fecal antigens.

Regarding therapy, empiric antibiotics were administered to nine patients (3%) with a pathological urine dipstick and discontinued in seven patients when a negative urine culture was obtained. The remaining two patients with documented urinary tract infections (UTI) continued antibiotic therapy based on antibiotic susceptibility tests. Antiviral drugs were not administered due to the overall favorable clinical course.

The hospitalization rate was 71% (195/274), with 83% in infants aged <29 days, and 67% in infants aged ≥29 days. The median LOS was 48 h in both age groups (Table 1). No patients required hospitalization in the PICU or NICU; only one patient required respiratory support with high-flow nasal cannula (HFNC). Ten patients required low-flow oxygen supplementation upon admission, but none required supplementation during the hospital stay. No secondary SBI were observed, and all hospitalized patients were discharged without evidence of short-term sequelae. No deaths occurred, and none of the patients returned to PED within a month of discharge. Additionally, no cases of Multisystem Inflammatory Syndrome (MIS-C) were registered among the patients included in the study. Differences between hospitalized and non-hospitalized patients are summarized in Table 6. In multivariable analysis, independent factors for hospitalization were fever ($p = 0.002$), poor feeding ($p = 0.015$), and lower age ($p < 0.001$). Table 7 reports possible explanatory

variables involved in prolonging LOS: only procalcitonin (PCT) higher than 0.5 ng/dl at arrival remained significant ($p = 0.036$) in the multivariable model.

Table 6. Univariate and multivariate analysis of independent factor assessed at the time of PED presentation in hospitalized versus non-hospitalized patients. Categorical variables are described as absolute and relative frequencies. GBS = group B Streptococcus; LRT = lower respiratory tract; URT = upper respiratory tract; WBC = white blood cells; ANC = absolute neutrophil count; CRP = C-reactive protein; PCT = procalcitonin; * Haldane-Anscombe correction.

	Hospitalization		**Univariable**		**Multivariable**	
	No	**Yes**	***p***	**Odds Ratio (95% CI)**	***p***	**Odds Ratio (95% CI)**
Sex			0.893	1.06 (0.628–1.79)		
Female	36 (29.5%)	86 (70.5%)				
Male	43 (28.3%)	109 (71.7%)				
Maternal positive GBS	3 (17.6%)	14 (82.4%)	0.572	1.79 (0.499–6.45)		
Comorbidities	4 (17.4%)	19 (82.6%)	0.457	1.68 (0.548–5.13)		
Older sibling	11 (14.5%)	65 (85.5%)	0.546	1.31 (0.579–2.98)		
Poor clinical condition	0 (0)	8 (100%)	0.11	7.12 (0.406–125) *		
Fever	54 (25.8%)	155 (74.2%)	0.06	1.79 (0.997–3.23)	0.002	2.79 (1.44–5.39)
Dyspnea	0 (0)	16 (100%)	0.008	14.6 (0.866–247) *	0.986	/
URT symptoms	24 (25.8%)	69 (74.2%)	0.482	1.25 (0.715–2.2)		
LRT symptoms	1 (5.3%)	18 (94.7%)	0.017	7.93 (1.04–60.5)	0.128	5.43 (0.61–47.98)
Vomiting	0 (0)	5 (100%)	0.326	4.59 (0.251–84.0) *		
Diarrhea	7 (50%)	7 (50%)	0.125	0.383 (0.130–1.13)		
Poor feeding	9 (15%)	51 (85%)	0.009	2.75 (1.28–5.91)	0.015	2.78 (1.21–6.35)
Cutaneous rash	1 (14.3%)	6 (85.7%)	0.677	2.48 (0.293–20.9)		
WBC > 10,000/mmc	0 (0)	15 (100%)	0.213	5.69 (0.323–100) *		
ANC > 5000/mmc	1 (16.7%)	5 (83.3%)	0.58	0.745 (0.08–6.85)		
CRP > 2 mg/dL	0 (0)	1 (100%)	1	1.10 (0.05–22.4) *		
PCT > 0.5 ng/mL	0 (0)	2 (100%)	1	1.04 (0.04–22.9) *		
Age (days, median)	57	42	<0.001	0.98 (0.968–0.992)	<0.001	0.976 (0.964–0.989)

Table 7. Univariate and multivariate analysis of independent factor assessed at the time of PED presentation in different LOS. LOS = length of stay; GBS = group B Streptococcus; LRT = lower respiratory tract; URT = upper respiratory tract; WBC = white blood cells; ANC = absolute neutrophil count; CRP = C-reactive protein; PCT = procalcitonin.

	LOS	**Univariable**		**Multivariable**	
	(h, Median)	***p***	**Estimate (95% CI)**	***p***	**Estimate (95% CI)**
Sex (male)	48	0.183	4.48 (−14.8–2.85)		
Maternal positive GBS	84	0.020	20.3 (3.29–37.3)	0.154	19.10 (−7.35–45.6)
Comorbidities	72	0.051	14.7 (−0.06–29.4)		
Older sibling	48	0.246	6 (−4.18–16.2)		
Poor clinical condition	108	<0.001	46.2 (25.2–67.3)	0.190	25.14 (−12.77–63.1)
Fever	48	0.629	−2.69 (−13.6–8.26)		
Dyspnea	84	0.003	23.8 (8.21–39.4)	0.979	−0.443 (−34.22–33.3)
URT symptoms	72	0.185	6.18 (−2.97–15.3)		
LRT symptoms	96	<0.001	26.1 (11.4–40.7)	0.139	28.47 (−9.5–66.4)
Vomiting	72	0.338	13.4 (−14.2–41)		
Diarrhea	48	0.441	−9.19 (−32.7–14.3)		
Poor feeding	48	0.006	13.8 (3.99–23.7)		
Cutaneous rash	60	0.854	2.37 (−23–27.7)		
WBC > 10,000/mmc	72	0.210	11.3 (−6.46–29)		
ANC > 5000/mmc	48	0.249	−17 (−46–12.1)		
CRP > 2 mg/dL	96	0.183	24.7 (−11.9–61.4)		
PCT > 0.5 ng/mL	120	0.012	58.2 (13.3–103.1)	0.020	54.64 (9.07–100.2)

4. Discussion

This study provides clinical insights into one of the largest series of children aged ≤ 90 days admitted to a tertiary PED with a confirmed SARS-CoV-2 infection. Our findings suggest that these patients commonly experience mild or asymptomatic course of the disease, with moderate-to-severe respiratory symptoms such as respiratory distress and apnea, occurring in only a minority of patients (6%), according to previously published reports [3,17,18]. Furthermore, the patients follow a benign clinical course, with unremarkable inflammatory blood test results, often not requiring hospital admission or specific therapies.

4.1. Clinical Presentation

Currently, a significant knowledge gap persists regarding the manifestation of the disease, clinical course, and risk factors for severe disease in neonates and infants infected with SARS-CoV-2 [2,17]. Some authors have suggested a potentially higher risk of severe disease in this age group compared to older children [3,4]. However, most of these studies only considered data on hospitalized infants, leaving the complete disease spectrum in this age group only partially explored [17].

Within our series, fever emerged as the most common presenting symptom of COVID-19 in infants (76%), followed by upper respiratory tract (URT) symptoms (34%) and poor feeding (22%) [3,9,15–18]. In contrast to other reports, we observed a lower frequency of gastrointestinal symptoms. Although it is often associated with fever or URT symptoms, poor feeding can be the only symptom observed. Thus, COVID-19 should also be considered in infants with inadequate milk intake [9,17,19].

We reported a non-negligible percentage (7%) of asymptomatic patients who accessed PED for reasons other than infectious symptoms such as trauma or parental concern due to contact with COVID-19, reinforcing the notion of a benign clinical course. In total, 9% of the patients in our series had comorbidities, with an equal distribution between the two age groups. Although comorbidities may influence the decision to hospitalize, especially in neonates, their disease course was unremarkable, as observed in other series [20,21].

4.2. Blood and Urine Test Findings

We found nine pathological urine dipsticks, and among these, we confirmed only two cases of UTIs with positive urine cultures, representing 1% of all febrile patients, consistent with a previously reported case series [22]. Seven out of nine patients with false-positive urine dipsticks received unnecessary antibiotic treatment. Based on these data and previous studies, it is advisable to consider UTIs even in febrile patients with a positive SARS-CoV-2 swab and without other clinical symptoms [14,23]. However, maintaining a high clinical suspicion is crucial to avoid misdiagnosis of UTIs, depending on the urine collection method, and unnecessary treatments [14,24,25].

We observed a higher percentage of blood tests performed in febrile newborns compared to infants (100% vs. 87%, $p = 0.013$), consistent with main recommendations advocating for more cautious clinical management in those under 28 days of life [13,14].

In addition to absolute values, we considered the cut-off values suggested for white blood cells (WBC), absolute neutrophil count (ANC), C-reactive protein (CRP), and PCT by previous authors [10,13,14,16].

However, blood test findings were largely inconsequential, showing no significant differences in absolute values across various groups, except for a slightly higher WBC count in neonates with more than six hours of fever.

Lower WBC and ANC values were detected in the infant group, both within and after six hours of fever (Tables 2 and 3). This can be partly explained by physiologically lower ANC values beyond neonatal age [18]. Furthermore, this was not associated with a worse prognosis.

No significant differences were found when comparing febrile and afebrile patients, suggesting a mild inflammatory state in COVID-19 patients and supporting a benign clinical course (Tables 4 and 5).

Uniquely, our study utilized cut-off values of the main inflammation indices suggested by the AAP and Step-by-step in a COVID-19 case series [13,14,16]. Considering the pathological values of CRP > 2 mg/dL, we observed worse values in infants with fever within six hours, suggesting a non-progression of the inflammatory state in the hours following fever (Tables 2 and 3).

Given the known high prevalence of SBI in children aged ≤90 days and the understanding that inflammatory markers performed within 12 h of fever may not differentiate between common benign viral infections and SBI, it is noteworthy that we did not detect any secondary SBI [10,13,14]. In light of our results and literature data, we suggest that laboratory tests in COVID-19 infants should not be routinely performed except in selected cases (poor clinical condition, poor feeding, comorbidities, and clinical concerns) [11]. Finally, the tests should not be repeated if the initial results fall within the normal range.

4.3. Management and Outcome

Five infants presented a coinfection with RSV and required oxygen supplementation, and one patient developed acute bronchiolitis with respiratory distress, requiring respiratory support with HFNC. This highlights the importance of considering alternative diagnoses beyond COVID-19, especially in cases of respiratory failure or the need for respiratory support, and the possibility of coinfections with more aggressive respiratory viruses [19,26].

Given the absence of specific guidelines, our standard practice was to hospitalize most positive infants and newborns for observation, following other guidelines for managing fever in patients aged ≤90 days [1,2,10,14]. This inclination is underlined by our multivariable analysis, where fever and lower age, along with poor feeding, emerged as independent factors influencing hospitalization. This is further confirmed by the higher hospitalization rate in neonates (p = 0.018), despite presenting a benign clinical course without complications, with an average length of hospital stay comparable to the infant group (p = 0.94) [3,17].

All our patients rapidly improved and were discharged, suggesting a favorable outcome of community-acquired COVID-19 in newborns and infants [3,6,27]. A PCT higher than 0.5 ng/mL upon admission was the only factor correlated with a longer LOS, potentially reflecting a more precautionary approach in patients with positivity of this inflammation marker that is universally correlated with sepsis [13,14].

The majority of hospitalized children were healthy, with no underlying clinical conditions, and were admitted primarily due to concerns related their young age, as reported in other case series [2,3,6,17–19,27,28].

Our data suggest that children <90 days old with COVID-19 are in good health, do not require hospitalization or specific care, and can, therefore, be easily managed at home [1,28,29]. Moreover, COVID-19 in infants under 90 days of age is milder than other acute viral illnesses in the same age group regarding the risk of developing bronchiolitis and/or the need for oxygen supplementation [19].

Therefore, we believe that a patient in good clinical condition with a positive swab for SARS-CoV-2 could be safely discharged without undergoing a full sepsis work-up [2,11,18]. In agreement with previous studies, we recommend that hospitalization should be considered in conditions that may lead to a more complicated clinical course or suboptimal home management, such as low-income family compliance or concern, low respiratory tract involvement, oxygen supplementation at admission, RSV coinfection, or poor feeding [17–19,30,31].

Additionally, hospitalization should also be recommended in case of underlying comorbidities or in case of prematurity <37 weeks of gestation, as these patients may

be at a higher risk of severe respiratory disease, and a greater need for ICU admission [3,17,18,20,21,32,33].

Similarly, hospitalization should be advised for unwell patients at the time of admission due to reported anecdotal cases of myocarditis and encephalitis related to SARS-CoV-2, even in the neonatal and childhood periods [2,34].

Figure 2 summarizes the suggestions provided in the text.

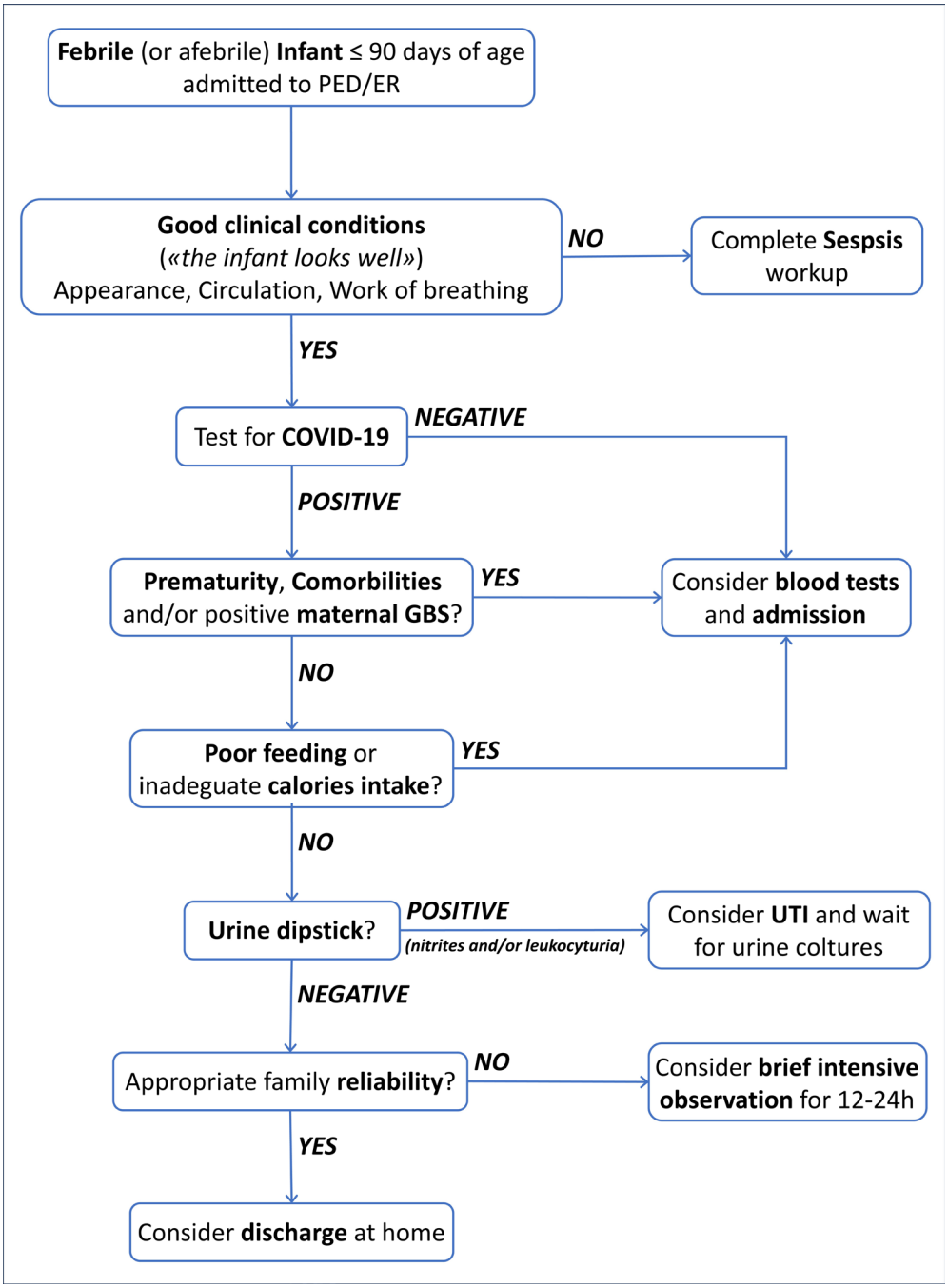

Figure 2. Shows our proposed management pathway, summarizing the previous suggestions.

5. Limitations

Our study's retrospective and monocentric design may introduce bias, as our findings may not be generalizable to other settings. Missing data for certain demographic items can impact sample representativeness and reduce the statistical power of our analysis, introducing bias in parameter estimation. Furthermore, the absence of complete data on

long-term follow-up beyond 30 days after discharge prevents us from drawing conclusions regarding long-term COVID-19 complications.

6. Conclusions

The lack of information on COVID-19 characteristics in neonates and infants aged <90 days remains a challenge for PED physicians. In our study, these patients experienced a non-specific, milder, or asymptomatic form of COVID-19. In addition, blood tests were consistent with a mild inflammatory response, emphasizing the need for such tests only in children presenting with noticeable illness [5,27].

According to our findings, precautionary hospitalization of infants who do not display signs of illness or without comorbidities may not be justified. Infants under 90 days of age could be treated like those with any other viral infections and be discharged to a reliable family with appropriate follow-up. Further larger prospective studies are necessary to confirm our findings and design a unique clinical management strategy for newborns and infants with COVID-19.

Finally, our research suggests that further studies are needed to integrate common algorithms for febrile infants with rapid viral infection diagnostic tests to reduce unnecessary hospitalizations.

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CLINICAL RESEARCH ARTICLE

Check for updates

Early diagnosis of serious bacterial infection in febrile infants using type I interferon signature

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BACKGROUND: Febrile infants ≤90 days are at high risk of serious bacterial infections, but prompt differentiation between viral and bacterial infections remains a challenge. Host gene expression signatures, particularly those involving the type I interferon (IFN) pathway, show promise as diagnostic tools. This study evaluates the ability of IFN signature to differentiate bacterial from viral infections in this population.

METHODS: This is a prospective, single-center study. Infants ≤90 days were enrolled. All patients underwent complete blood count, CRP, PCT, and IFN signature, assessed by analyzing the expression of six interferon-stimulated genes. Based on routine investigations, patients were divided into bacterial (BI, $n = 8$) and non-bacterial infection (non-BI, $n = 39$) and healthy controls (HC, $n = 3$). Regression analysis was used to determine the variable's predictive correlation.

RESULTS: Forty-seven febrile infants were enrolled. IFN signature resulted significantly elevated in non-BI compared to BI ($p < 0.001$) and HC ($p = 0.008$), even within 12 h of fever onset. IFN demonstrated the highest AUC for predicting BI. Combining IFN with CRP or PCT yielded the most robust predictive models (AUC = 0.99 and 0.98).

CONCLUSIONS: IFN signature resulted as a promising, reliable predictor of BI versus non-BI in febrile infants. This novel biomarker, combined with others, could improve the management of febrile infants reducing unnecessary antibiotics and hospitalizations.

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IMPACT:

- This is the first study to focus on the IFN signature in febrile infants under 90 days old, adding novel insights into the potential of host immune responses as diagnostic biomarkers.
- This study demonstrates that the peripheral blood interferon signature, specifically the expression of six IFN-stimulated genes, can distinguish bacterial from non-bacterial infections in febrile infants aged 90 days or younger in the very first hours of fever.
- The IFN score, when combined with traditional inflammatory markers, offers high sensitivity and specificity, making it a potential diagnostic tool for optimizing treatment and reducing unnecessary interventions.

INTRODUCTION

Febrile infants aged 90 days or younger are at substantially higher risk of serious and invasive bacterial infections (SBIs and IBIs, respectively) compared with older children due to the lack of vaccination protection, immaturity of their immune systems, and potential perinatal exposure to certain pathogens.[1–6] Moreover, fever may be the only symptom of a significant underlying infection in this age group.[1–6]

Given these substantial risk factors, management of this population frequently involves lumbar puncture, broad-spectrum antibiotic administration, and hospitalization, although viral infections may represent up to 85% of the causes of fever.[7] This undoubtedly has a negative impact on antimicrobial resistance, microbiota of this young population, hospital-related complications, healthcare costs, and patients' and family well-being.[8,9] Identifying a small number of infants with SBI/IBI among those who present to both Adult and Pediatric Emergency Department (PED) with self-limiting viral infections remains a major challenge for frontline clinicians, especially given that the clinical presentation is often non-specific and disease progression can be very rapid in this age group.[1–5,7]

Many efforts have been made to develop risk-stratification tools to early delineate which febrile infants require extensive testing and empirical treatment and which infants are at low risk and can be conservatively managed in an outpatient setting.[4,5,7–9] These approaches are based on specific predictors, such as clinical appearance, age, and laboratory test results, and have shown high sensitivity, although with lower specificity.[5,7,10–13]

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In recent years, a growing body of evidence has suggested that microbial pathogens induce specific host responses or "RNA biosignatures" that can be identified using microarray analyses of blood leukocytes.[14–16] These studies have demonstrated the value of the host's peripheral blood gene expression response signatures in accurately discriminating bacterial, viral, and non-infectious etiologies, suggesting that it can represent a diagnostic strategy complementary to those already in use, with some emerging evidence in young infants.[6,14–21]

Interferons (IFNs) are cytokines involved in host immune response to pathogens, and reported robust signatures capable of discriminating between bacterial and viral infections include members of the type I IFN pathway.[15,22–30]

The objective of this study was to evaluate the ability of the peripheral blood IFN signature to distinguish bacterial from viral infections in febrile infants aged 90 days or younger who presented to the PED.

METHODS

Population

This was an observational, prospective, single-center, non-profit study. All infants aged ≤90 days who presented at the IRCCS Giannina Gaslini third-level Children Hospital's PED with fever (axillary temperature ≥37.5 °C or rectal temperature ≥38 °C) observed at the time of medical assessment or found at home within 12 h prior to arrival were eligible for enrollment. Infants were excluded if they had received antibiotics within 48 h of PED presentation; if at least one measurement of complete white blood cell (WBC) count, absolute neutrophil count (ANC), C-reactive protein (CRP), or procalcitonin (PCT) was not obtained; if an RNA blood sample was not collected; or if a parent or guardian refused consent.

Study design and procedures

The data collected included patient age, gestational age, sex, medical history, and duration of fever. Standard laboratory investigations of febrile infants were performed, and a blood sample for IFN signature analysis was collected. Analysis and culture of urine, blood, and cerebrospinal fluid (CSF) were part of the standard evaluation for fever in these infants. However, blood and CSF cultures were not performed in all cases because of clinical judgment. We included in the study all those who underwent at least one determination of CBC, CRP, or PCT. Based on the results of laboratory tests (blood, urine, CSF, and nasopharyngeal swabs) and clinical course, the enrolled patients were categorized by emergency room pediatricians (E.F., G.V., M.B., T.B.), blinded to the IFN signature results, into two groups: those with bacterial infection (BI) and those without bacterial infection (non-BI).

IFN signature and IFN score

Peripheral blood samples (2.5 mL of blood) of subjects enrolled were collected in "PAXgene Blood RNA Tubes" and total RNA was purified using the "PAXgene Blood RNA Kit" (PreAnalytiX, CH). RNA samples were quantified and stored at −80 °C until analyzed. In all, 1 µg RNA was retro-transcribed using SuperScript VILO cDNA Synthesis Kit (Thermo Fisher), and cDNAs were stored at −20 °C. Real-time PCR was performed in duplicate using gene-specific custom-designed FAM probes (TibMolBiol, Roche, Germany).

IFN signature was assessed by calculating the expression of six IFN-stimulated genes (ISGs; IFI27, IFI44L, IFIT1, ISG15, RSAD2, and SIGLEC1). A synthetic oligonucleotide containing the regions of the six ISGs and two housekeeping genes (IFI27, IFI44L, IFIT1, ISG15, RSAD2, SIGLEC1, TBP, and HPRT1) was used as a reference to calculate standard curves.[31] Cycle threshold values were normalized to the geometric means of two internal controls (TBP and HPRT1) and expressed as the gene score (GS). IFN score was calculated as the geometric mean of the GS of the six ISGs. Laboratory staff performing gene expression analyses (P.B., S.V.) were blinded to clinical data and final diagnoses.

Study definitions and outcome measures

Bacteremia and bacterial meningitis were defined as the growth of a single pathogen in the blood or CSF, respectively. Urinary tract infection (UTI) was defined as the growth of a single pathogen in the urine with colony counts (colony-forming units (CFUs)) meeting the criteria of ≥100,000 CFUs/mL in association with an abnormal dipstick test or urinalysis (positive for leukocyte, leukocyte esterase, or nitrites). Blood and CSF cultures were considered to reflect the growth of contaminants when bacterial isolates were not commonly accepted pathogens. We defined SBI as the presence of bacterial meningitis or bacteremia or UTI and IBI as the presence of bacterial meningitis or bacteremia.

Patients were classified as having bacterial infections (BI group) if they turned out to have a SBI, otherwise, they were assigned to the non-bacterial infection (non-BI) group. To detect viral etiological agents, multiplex PCR assays or rapid antigen tests were performed on nasopharyngeal swabs and/or antigen testing of stool samples depending on the clinical context.

Patients who did not undergo culture testing were classified as non-BI if they exhibited a favorable and self-limiting clinical course with spontaneous defervescence during at least 24 h of in-hospital monitoring without antibiotic administration, even if a viral etiology was not ultimately identified.

Patients in whom no pathogen was identified were classified as non-BI if all cultures performed prior to antibiotic administration were negative, or if they experienced a favorable and self-limiting course with spontaneous defervescence and recovery during hospitalization without antibiotic therapy.

Data analysis

We compared the demographics, laboratory results, and IFN scores of the BI, non-BI, and HC groups. The data were organized in tabular form and analyzed anonymously. Comparisons between categorical variables were performed using cross-tabulations, and statistical significance was assessed using two-sided Fisher's exact test. For comparisons of multiple independent variables, the Kruskal–Wallis test was employed, followed by Dunn's test for pairwise multiple comparisons. *P* values for each comparison were adjusted using the Benjamini–Hochberg correction for multiple testing. For comparison of continuous variables between the two groups, the choice between parametric (two-sided Student's *t* test) and non-parametric (two-sided Mann–Whitney *U*-test) tests was based on the data distribution and sample size. A logistic regression model was employed to predict the probability of bacterial or non-BI infections based on a set of predictor variables. Model fit was assessed using the area under the receiver operating characteristic curve (AUC-ROC). All statistical analyses were performed using GraphPad Prism version 9.1.0 for Windows (GraphPad Software, San Diego, California, www.graphpad.com).

RESULTS

Study population

Forty-nine febrile neonates and infants aged ≤90 days who presented to the PED of Gaslini Children Hospital were enrolled in the study over a 12-month period. Two subjects were excluded from subsequent analyses because their RNA samples resulted in low quality. Three healthy infants were enrolled as controls. Figure S1 reports the inclusion–exclusion criteria.

The median age was 40 days (95% confidence interval (CI) 32–47 days), and 40% ($n = 20$) of the participants were female. The age distribution was as follows: 22 patients aged ≤28 days (44%), 15 patients 29–60 days (30%), and 13 patients 61–90 days (26%). Of the 47 febrile subjects enrolled, 8 (17%) were assigned to the BI group, while 39 (83%) were assigned to the non-BI group (Fig. S1). We did not detect any viral–bacterial co-infection.

There were no significant differences in age or sex between the two groups and healthy controls (HC) (Table 1).

Bacterial infections

Eight infants (17%) had a BI. Of these, five had IBIs with isolated bacteremia, and three had UTI without bacteremia. No case of bacterial meningitis was observed. IBIs were identified in 2 of 22 infants aged 28 days and younger (9%), in 3 of 15 infants aged 29–60 days (20%), and in none of the infants aged 61–90 days. BIs and pathogens are listed in Table S1.

Non-BIs

Thirty-nine infants (83%) were classified as having non-BI infections. Of these, 19 had no identified pathogen. In 19 patients, a viral pathogen was detected among *parechovirus, enterovirus,*

Table 1. Demographic characteristics.

	Overall ($n = 50$)	Non-BI ($n = 39$)	BI ($n = 8$)	HC ($n = 3$)	p Value
Age (days)	40 (32–47)	40 (31–49)	42 (23–62)	31 (10–69)	0.71
Female	20 (40%)	17 (44%)	2 (25%)	1 (33%)	0.60
Age ≤28 days	22 (44%)	17 (44%)	3 (38%)	2 (67%)	
Age 29–60 days	15 (30%)	12 (31%)	3 (38%)	0 (0%)	
Age 61–90 days	13 (26%)	10 (26%)	2 (25%)	1 (33%)	

Quantitative variables are presented as means with 95% confidence intervals. Qualitative variables are presented as absolute frequencies and corresponding percentages within their respective columns. p Values were calculated using the Chi-square test for qualitative variables and the Kruskal–Wallis test for quantitative variables.
OR odds ratio, *MD* mean difference.

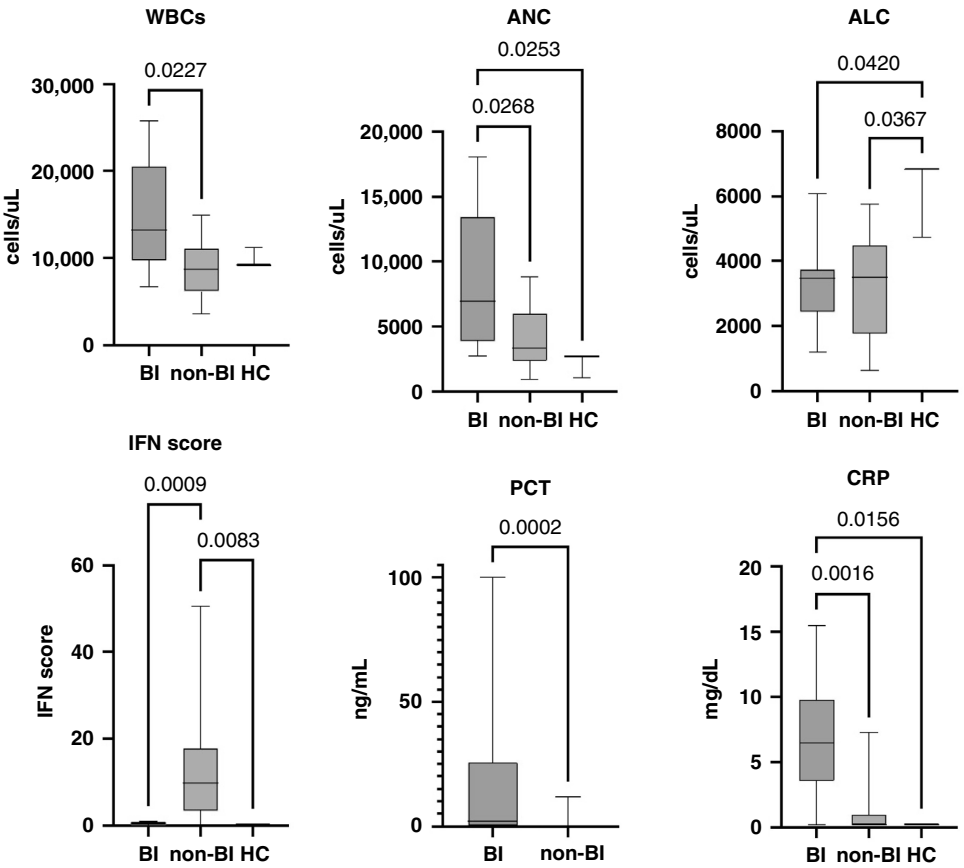

Fig. 1 Comparison of inflammatory markers between bacterial infection (BI), non-bacterial infection (non-BI), and healthy controls (HC) groups. The box bounds the IQR divided by the median, and the whiskers extend to the 5th and 95th percentiles. p Values were assessed through Dunn's test and adjusted using the Benjamini–Hochberg correction for multiple tests (p value for PCT was instead calculated using Mann–Whitney U-test). In the figure, only statistically significant p values ($p < 0.05$) are shown. WBC white blood cell, ANC absolute neutrophil count, ALC absolute lymphocyte count, CRP C-reactive protein, PCT procalcitonin.

adenovirus, metapneumovirus, parainfluenza virus, SARS-CoV-2, and *respiratory syncytial virus (RSV)* (Table S1). A single patient was diagnosed with a *Candida glabrata* UTI.

Inflammatory markers

CRP levels were significantly higher in the BI group than in the non-BI ($p = 0.002$) and HC groups ($p = 0.02$). PCT levels were also significantly higher in the BI group than in the non-BI group ($p < 0.001$). PCT levels were not measured in the HC group. Analysis of the leukocyte count revealed a significant increase in the WBC count in the BI group compared to that in the non-BI group ($p = 0.02$). ANC was significantly higher in the BI group compared to both the non-BI and HC groups ($p = 0.03$ and $p = 0.03$, respectively). Interestingly, the absolute lymphocyte count (ALC) was significantly decreased in both the BI and non-BI groups compared to the HC group ($p = 0.04$ and $p = 0.04$, respectively) (Fig. 1, Table S3).

IFN score

IFN score differed significantly among the three groups ($p < 0.001$). Specifically, the IFN score was elevated in the non-BI group compared to both the BI and HC groups ($p < 0.001$ and $p = 0.008$, respectively). No significant difference was observed between the HC and BI groups (Fig. 1).

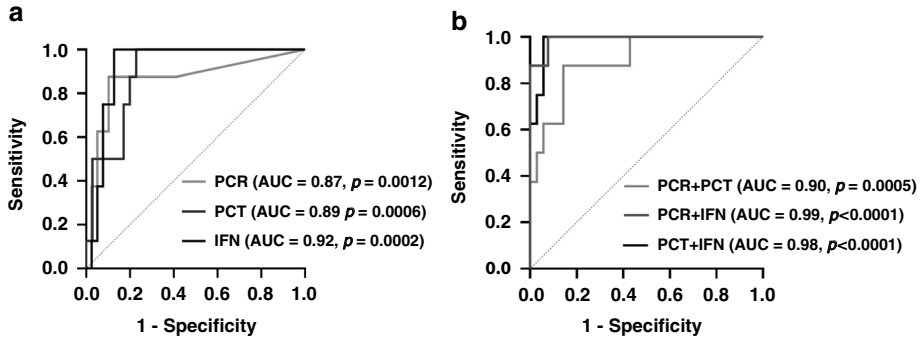

Fig. 2 ROC curve analysis of inflammatory markers for bacterial infection prediction. a shows the predictive performance of CRP, PCT, and INF score as individual markers for bacterial infection; **b** illustrates the performance of these markers when evaluated in pairwise combinations, with the specific pairings shown in the figure. *p* Values were calculated using logistic regression models. CRP C-reactive protein, PCT procalcitonin, AUC area under the curve.

Comorbidities

Regarding comorbidities, a significantly higher proportion of subjects in the BI group (75%) had comorbidities compared to the non-BI group (10%) ($p < 0.001$). This is consistent with the general consensus that patients with pre-existing comorbidities and risk factors are at an increased risk of SBI. A comprehensive list of the identified comorbidities is provided in Table S4.

When comparing CRP, PCT, WBC, ANC, and IFN scores between patients with BI ($n = 8$) and those with viral infection and pre-existing comorbidities ($n = 3$), PCT and IFN scores showed statistically significant differences between the two groups ($p = 0.01$ and $p = 0.01$, respectively; Mann–Whitney *U*-test), whereas no significant differences were found for the other markers.

Logistic regression models

Using logistic regression models, we analyzed the performance of CRP, PCT, and IFN scores in predicting the presence of BIs, both individually and in combination. Our analysis revealed that all three markers were sensitive and specific when considered alone; notably, the IFN score demonstrated the highest AUC (0.92, 95% CI 0.85–1.00) compared to CRP (0.87, 95% CI 0.70–1.00) and PCT (0.89, 95% CI 0.80–0.99) (Fig. 2a). When analyzing the performance of markers in pairs, models utilizing the IFN score in combination with PCT or CRP showed superior performance compared to models using only CRP and PCT. Specifically, the IFN + CRP model exhibited the best performance (AUC 0.99, 95% CI 0.97–1.00), followed by the IFN + PCT model (AUC 0.98, 95% CI 0.95–1.00). The CRP + PCT model achieved an AUC of 0.90 (CI 95% 0.79–1.00), which was lower than the IFN score alone (Fig. 2b).

Inflammatory markers and duration of fever

When analyzing the blood tests obtained within the first 6 h of fever ($n = 9$; BI, $n = 2$; non-BI, $n = 7$), no significant differences were found between the groups for traditional inflammatory markers (CRP, PCT, WBC, ANC, and ALC). The IFN score could not be assessed within this time window owing to insufficient data. Of note, a trend towards significance was detected for PCT ($p = 0.056$) when comparing the BI and non-BI groups.

When analyzing the data obtained within the initial 12 h of fever onset, significant differences were observed in the IFN score ($p = 0.03$), CRP ($p = 0.04$), PCT ($p = 0.02$), WBC ($p = 0.03$), and ANC ($p = 0.006$) values between the BI and non-BI groups (Fig. 3).

Viral infections and IFN score

We compared IFN scores among the different viruses identified in our sample to assess whether there were variations in IFN pathway induction and found no significant differences (Fig. S2).

Predictive probability (PP) of BI of different logistic regression models

We subsequently analyzed a cohort of subjects who eventually tested negative for BI but had received empirical antibiotic therapy. Among the 39 patients in the non-BI group, 14 (36%) received empirical antibiotic therapy. Of these, five were positive for Parechovirus, three for enteroviruses, one for both enterovirus and adenovirus, one for RSV, and one for rhinovirus. In three patients, the pathogen was not identified. Applying the logistic regression models previously analyzed to this cohort, the PP of BI based on the IFN score was found to be <0.1% in 10 out of 14 of these patients and 1.4% in another; when applying the IFN + CRP score model, the PP of BI improved further resulting ≤0.3% for 13/14 subjects. The CRP + PCT model was the least effective in identifying BIs (Table S5). The mean age of this cohort was 17 days, and 12 of the 14 patients were <28 days old.

DISCUSSION

To our knowledge, this is the first study to analyze the type I IFN signature in febrile infants aged 90 days or younger. Our findings primarily support the evidence that these patients, despite their young age, are capable of mounting a robust and differentiated innate inflammatory response against various microbial agents, consistent with the literature.[6,21–23,26]

Our data demonstrate that the IFN score is significantly higher in non-BIs in this population, whereas no differences in its value were found between the BI group and HC. These findings align with previous literature, which has consistently shown that the IFN pathway is activated during viral infections but not during bacterial ones.[15–23,25–27]

In our logistic regression models, the IFN signature alone demonstrated high sensitivity and specificity in discriminating between BI and non-BIs, suggesting its potential as a useful tool when combined with traditional inflammatory markers. Similar findings have been previously reported in the literature for populations older than our cohort.[25]

In our analysis of non-BI patients who received empirical antibiotic therapy, we observed that our IFN score model yielded exceptionally low PP for BIs (<1.4% in 11/14); combining the IFN score with CRP further improved the model's performance, reducing the probability to <0.3% in 13/14 patients.

These findings suggest that the IFN signature may be a valuable tool for optimizing therapeutic decisions in febrile infants. However, to accurately assess the performance of the logistic regression models developed in our study population, they should be validated in an independent cohort.

Currently available inflammatory markers, combined with clinical evaluation, are insufficiently sensitive and specific for early

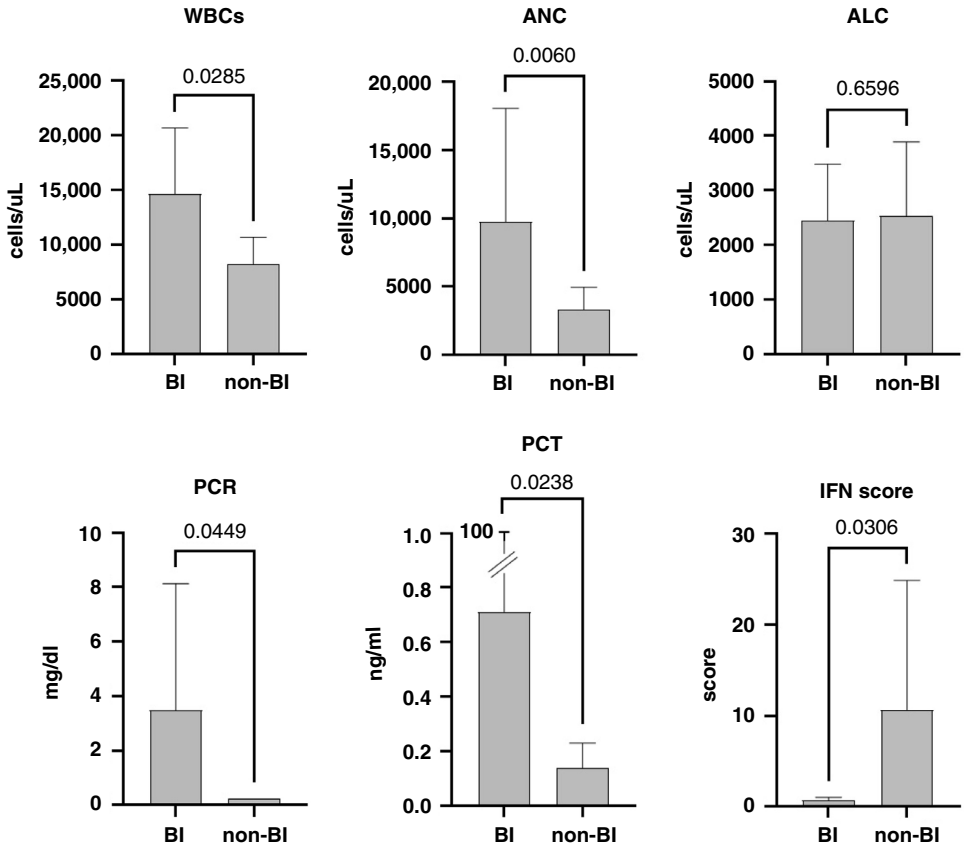

Fig. 3 Comparison of inflammatory markers and IFN score performed within 12 h of fever onset between bacterial infection (BI) and non-bacterial infection (non-BI) groups. The histograms show the medians with 95% CI. *p* Values are based on Mann–Whitney *U*-test. WBC white blood cell, ANC absolute neutrophil count, ALC absolute lymphocyte count, CRP C-reactive protein, PCT procalcitonin.

discrimination of SBI in this febrile young population.[1–7] Culture of bacteria from normally sterile sites, which is currently the gold standard for confirming an ongoing BI, takes several days for results to be obtained. Moreover, it could be negative when the infection resides in inaccessible sites or if antibiotics have been previously administered, and the false-positive rate in infants may be high.

In this context, information regarding the host immune response could provide an essential contribution to the early identification of SBIs. Our study demonstrated that the IFN score, both alone and when used as a complement to current markers, is capable of discriminating between BIs and non-BIs, giving the possibility of selecting patients who require targeted antibiotic therapy.

It is not uncommon for febrile infants aged 90 days or younger to present at PED for medical evaluation very early from the onset of fever, and it is well established that traditional inflammatory markers in this timeframe are unreliable in identifying SBI.[7,32,33]

Our findings are consistent with these conclusions. Indeed, in our cohort, no inflammatory markers assessed within the first 6 h of fever onset showed differences between the BI and non-BI groups, while they became significant when extending the interval from fever onset to the first 12 h (CRP, PCT, WBC, and ANC). We suggest the need to monitor patients with normal blood tests but fever onset of <6 h and repeat inflammatory markers after this time to have more accurate and reliable blood tests.[7,13,34]

Owing to insufficient data, we were unable to assess the IFN score within the first 6 h of fever, a time window where RCP and PCT in two patients belonging to the BI group had initially resulted negative. However, the test demonstrated reliability when performed within the first 12 h of fever onset. We believe it would be highly interesting to evaluate its performance when performed within the first hours of fever onset, considering that biologically the production of CRP and PCT follows cytokine activation and that there is evidence that host gene expression can detect a viral infection before the onset of symptoms.[30]

Limitations

This study has some limitations. First, its pilot nature, the small sample size, the absence of an independent validation dataset, and a limited number of HC do not allow to provide definitive recommendations on the use of the IFN score in clinical practice. Moreover, the absence of viral–bacterial co-infections in our cohort prevented us from evaluating IFN scores in this scenario, where the IFN pathway might be activated by viral infection independent of BI. Finally, a more rapid and cost-effective IFN score method may be mandatory to translate this result in the context of a point-of-care setting.

CONCLUSIONS

Early identification of febrile infants aged ≤90 days with SBI remains a major challenge for clinicians, despite their known low incidence. In this preliminary study, the analysis of six ISGs and the IFN score alone was sufficient to discriminate between BIs and non-BIs in this young febrile population. Moreover, when used in conjunction with traditional markers, the IFN score demonstrated increased sensitivity and specificity.

This might allow for a more accurate diagnosis and treatment of the patient, significantly reducing unnecessary antimicrobial therapies, invasive procedures, and hospitalizations.

Further prospective studies with larger populations are needed to refine and validate the IFN score, alone or associated with other inflammatory markers, for the optimal clinical-therapeutic management of febrile patients <90 days of life. Additionally, future research should investigate the feasibility of implementing IFN score testing using clinical assays suitable for hospital laboratories or point-of-care settings.

DATA AVAILABILITY

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

E.F., T.B., S.V., and R.P. conceived the idea; E.F., G.V., M.B., F.S., and E.P. collected the samples; P.B. performed the interferon signature analysis; E.F. and T.B. performed the experiments and analyzed the data; E.F. and T.B. prepared the first draft. S.V. and E.P. revised the manuscript. All the authors have read and agreed to the published version of the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Approval for this study was granted by the local Ethics Committee (CET - Liguria:262/2024 - DB id 13963). Informed consent was obtained from the parents of all patients.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41390-025-04229-0.

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