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SCUOLA DI SPECIALIZZAZIONE IN PEDIATRIA

TESI DI SPECIALIZZAZIONE

Evaluation of peripheral blood interferon signature as early indicator of viral infection in febrile infants aged 90 days or younger

Relatore
Prof. Mohamad Maghnie

Correlatori
Dott.ssa Emanuela Piccotti
Dott. Tommaso Bellini

Candidata
Dott.ssa Elena Fueri

A Luca,
l’amore che mi permetti di provare
è sempre stato la risposta.

Alla zia Maria,
perché se ne sono capace
è anche merito tuo.

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1.Background

1.1 Fever in infants under 90 days of age: risk of serious and invasive bacterial infections

Fever is the most common reason for medical consultation in the pediatric emergency department (PED) around the world[1].

Although in most cases the causative agent turns out to be a virus [2,3], febrile infants aged 90 days or younger are at substantial higher risk of severe and invasive bacterial infections (SBIs and IBIs) compared with older children due to the lack of vaccination protection, immaturity of their immune systems and potential perinatal exposure to certain pathogens, moreover, fever may be the only symptom of a significant underlying infection in this group of age.[3–7]

The definition of **serious bacterial infection (SBI)** remains controversial in the literature, the majority of studies included in this group urinary tract infections (UTIs), bacteremia, and meningitis, but there's no consensus on including pneumonia and bacterial gastroenteritis; on the other hand, the definition of **invasive bacterial infection (IBI)** is clear and accepted, it includes bacteremia and bacterial meningitis (isolation of a bacterial pathogen in a blood or cerebrospinal fluid culture).

According to the most recent literature, among febrile infants under 90 days of age, SBIs account for 8.4%-28% of cases and IBIs for 1.4-3.9%[6–10] with greater prevalence in the first month of life (Table 1 and Table 2).

Table 1. Prevalence of SBI in febrile infants aged 90 days or younger

Age (days)	Prevalence of SBI				
	Bonilla et al. [8]	*Aronson et al.* [6]	*Garcia et al.*[9]	*Gomez*[7]	*Brigadoi et al.*[11]
≤ 90	18,3%	8,4%	19,7%	23%	27.6%
< 21			30.5%		31.4%
< 28	21.5%	11.1%	27%		29.3%
29-60	16.6%	7.5 %	18.2%		22.7%
61-90	18.5%	7.7 %	17.5%		34.5%

Table 2. Prevalence of IBI in febrile infants aged 90 days or younger

Age (days)	Prevalence of IBI							
	Bonilla[8]	*Garcia*[9]	*Gomez*[10]	*Powell*[5]	*Biondi*[4]	*Cruz*[12]	*Aronson*[6]	*Gomez*[7]
≤ 90	2,5%	2,1%	1,4%				2,7%	3.9%
< 21		4.7%	4.6%	4 %				
< 28	5.1%	3.2%		3.1%	3% *bacteriemia* 1% *meningitis*	4,3%		
29-60	2.3%	2.6%		1.1%	1.6% *bacteriemia* 0.4% *meningitis*	1.3%		
61-90	1.1%	1.6%						
22-90		1.8%	1%					

1.2 Etiology of bacterial infections in febrile infants under 90 days of age

Since the 1980s, the etiology of bacterial infections in neonates and infants has changed as a result of many factors, including prenatal Group B streptococcus (GBS) screening and incorporation of immunization against Streptococcus pneumoniae and Haemophilus influenza. Furthermore, improvements in food safety may have resulted in a decrease in the incidence of disease caused by Listeria monocytogenes.[3]

Many and recent studies demonstrate that Escherichia coli is the most common organism to cause IBI under 90 days of age followed by GBS and that these bacteria are the two primary causative agents of bacterial meningitis in infants aged 0 to 90 days. Other microorganisms more frequently implicated in IBI in this group of age were found to be: enterococcus faecalis, streptococcus pneumoniae, staphylococcus aureus, streptococcus viridans, listeria monocytogenes.[3,5,7,8,13,14]

Considering febrile infants 60 days of age and younger, Powell et al. found that the main causative agent of IBI is Escherichia coli followed by da GBS, followed by Staphylococcus aureus and Enterobacter cloacae [5] and GBS resulted the primary cause of bacterial meningitis overall, these findings align with those reported by Cruz et al.[12] and by Woll et al.[15] even if in the latter one GBS predominates in the second month of life, not only as a cause of meningitis but also of bacteremia; Staphylococcus aureus remains the third most common pathogen, while Enterococcus, Klebsiella, and "other Gram-negative bacteria" were found to be more frequent than Enterobacter, with a different distribution between the first and second month of life.

1.3 Febrile infants under 90 days of age: current management

Given their substantial risk of SBI/IBI, the management of febrile infants younger than 90 days of age frequently involves lumbar puncture, broad-spectrum antibiotic administration, and hospitalization, although viral infections are the primary cause of fever. This undoubtedly has a negative impact on: antimicrobial resistance[16], microbiota of this young population, hospital-related complications and healthcare costs.

Identifying the small number of infants with SBI/IBI among the many who present to the PED with self-limiting viral infections remains a major challenge for front-line clinicians, especially given that the clinical presentation is often non-specific and disease progression can be very rapid in this age group.

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 the outpatient setting without lumbar puncture nor empirical antibiotic treatment; these approaches are based on specific predictors such as clinical appearance, age, and laboratory test results and they have shown high sensitivity, although lower specificity.[7,17–19]

Moreover, currently available diagnostic strategies for pathogen identification are limited by a long time to result and inability to discriminate infection from colonization.

The *Step-by-Step approach*, developed in Europe, and the *PECARN prediction rule*, developed by the North American Pediatric Emergency Research Network, are two recently and prospectively validated tools to identify low risk infants who can be managed without lumbar puncture (LP), antibiotics, or hospitalization (Table 3). [7,18]

Table 3. Risk-stratification tools for febrile young infants

		Sensitivity for IBI	Specificity for IBI	NPV for IBI
PECARN PREDICTION RULE [18] **Under 60 days of age (Internally validated)**	**Low risk if all criteria are met:** 1) Urinalysis negative for leukocyte esterase, nitrite and pyuria (≤ 5 WBC/hpf) 2) ANC ≤ 4090/uL 3) PCT ≤ 1,71 ng/mL	100% (95% CI 77.2 to 100)	60% (95% CI 56.6 to 63.3)	100% (95% CI 99.2 to 100)
STEP-BY-STEP APPROACH[7] **Under 90 days of age (Externally validated)**	**Low risk if all criteria are met:** 1) Well-appearing 2) 22 to 90 days old 3) Urinalysis negative for leukocytes 4) PCT ≤ 0.5 ng/mL 5) CRP ≤ 20mg/L and ANC ≤ 10000/uL	92% (95% CI 84.3 to 96)	46.9% (95% CI 44.8 to 49)	99.3% (95% CI 98.5 to 99.7)

ANC absolute neutrophil count; CRP C-reactive protein; IBI invasive bacterial infection; NPV negative predictive value; PCT procalcitonin; WBC/hpf white blood cells per high-powered field.

In 2021, the American Academy of Pediatrics (AAP) published a practice guideline: “Evaluation and Management of Well-Appearing Febrile Infants 8 to 60 Days Old.”[3] This guideline specifically applies to well-appearing, term, and previously healthy febrile infants and proposes three algorithms for management in the age groups of 8 to 21 days, 22 to 28 days, and 29 to 60 days (Figure 1-3). Inflammatory markers are considered abnormal at the following levels: procalcitonin >0.5 ng/mL, CRP >20 mg/L, and ANC >4000 to 5200 per mm3 and, only for 22 to 60 days group, temperature >38.5 °C. The guidelines indicates that HSV should be considered when: there is a maternal history of genital HSV lesions, fevers from 48 hours before to 48 hours after delivery, in infants with vesicles, seizures, hypothermia, mucous membrane ulcers, CSF pleocytosis in the

absence of a positive Gram stain result, leukopenia, thrombocytopenia, or elevated alanine aminotransferase levels.

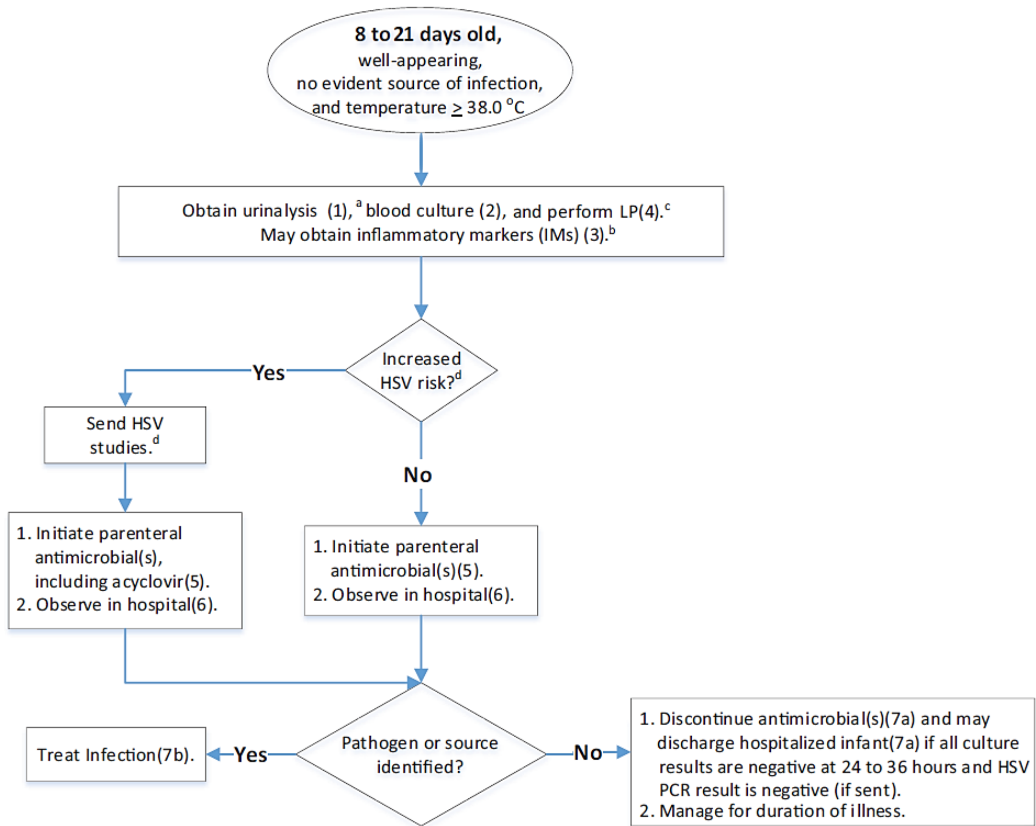

Figure 1. AAP Algorithm for 8- to 21-day-old febrile infants.
Figure from Pantell et al. [3]

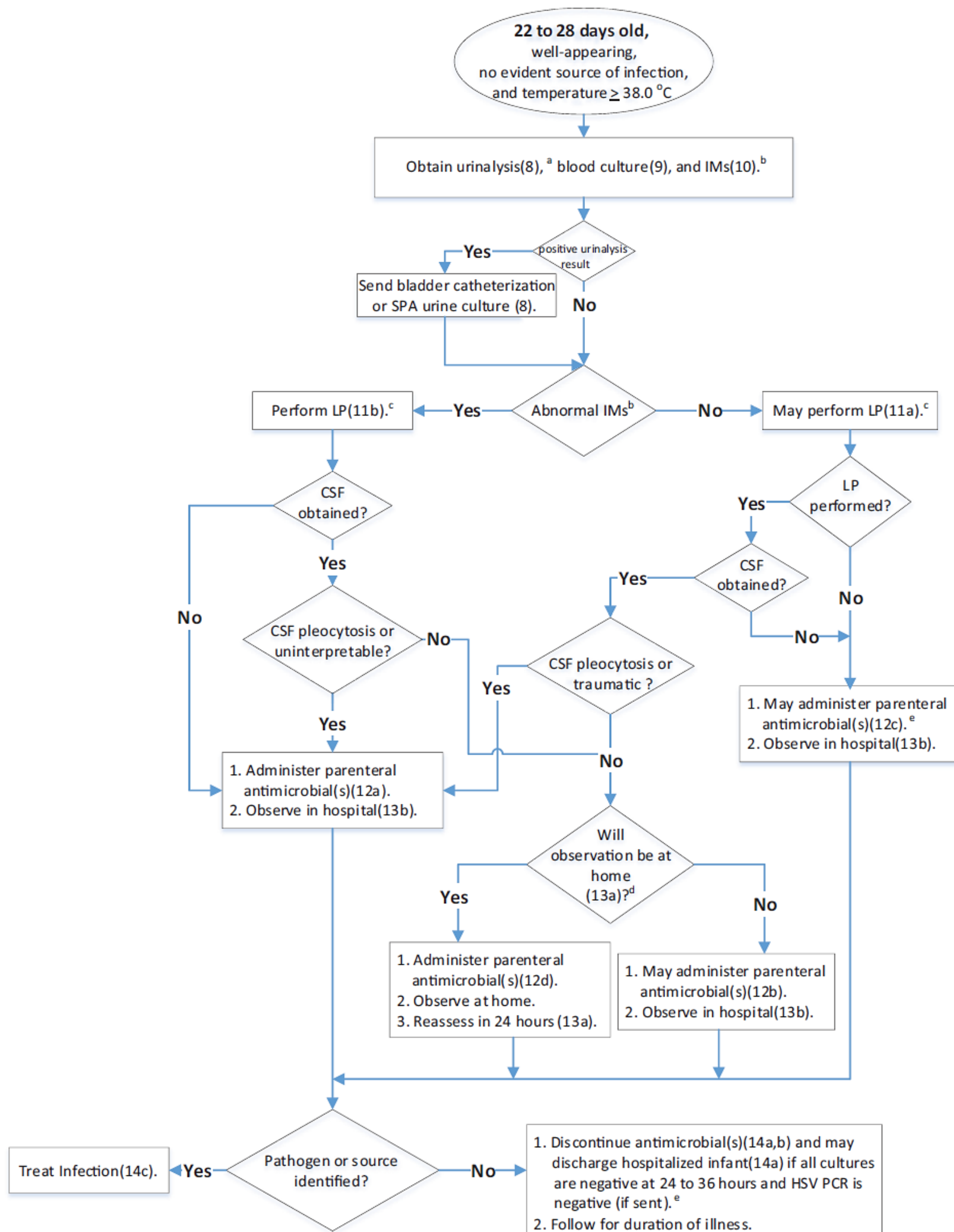

Figure 2. AAP Algorithm for 22- to 28-day-old febrile infants.
Figure from Pantell et al. [3]

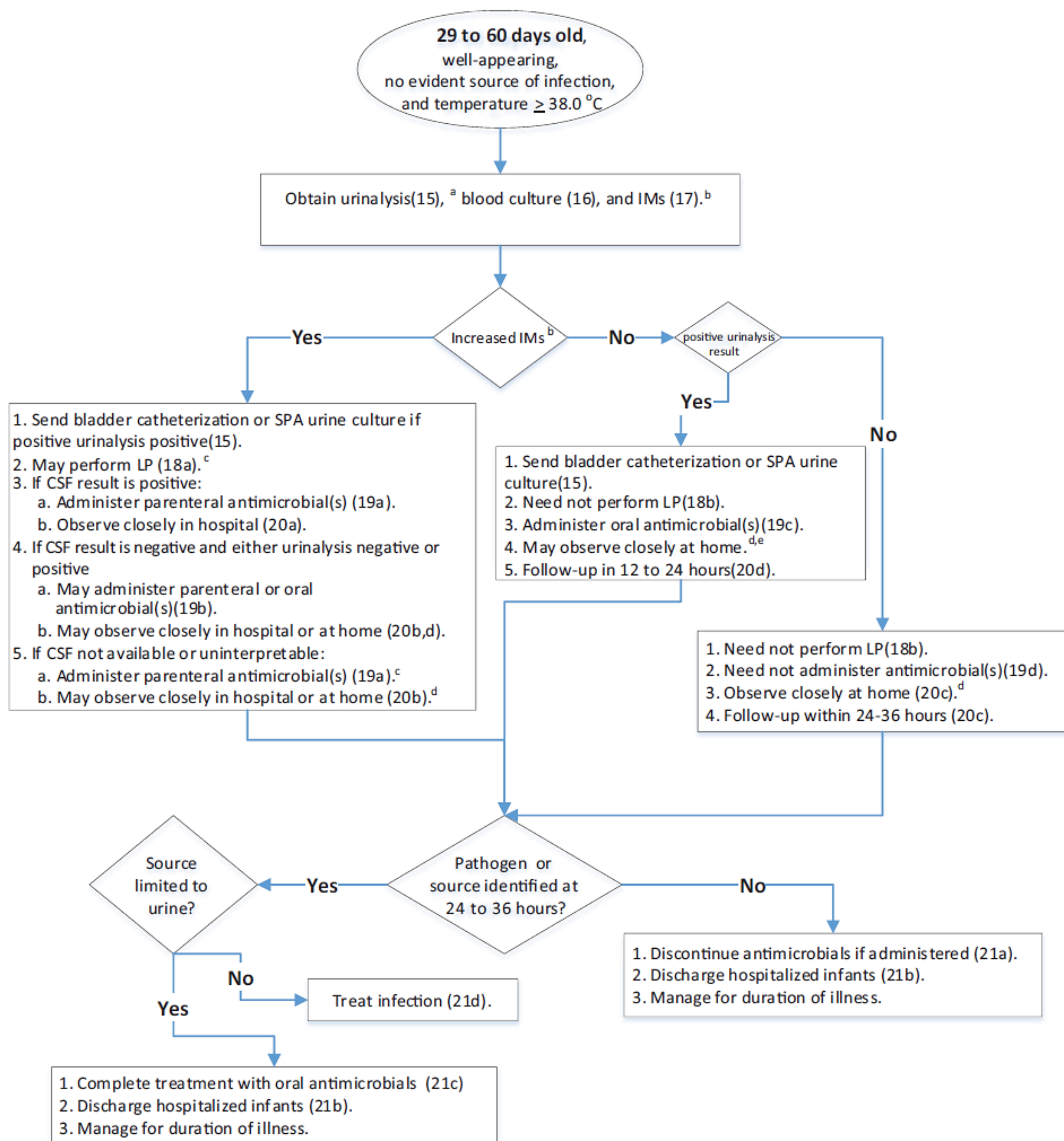

Figure 3. AAP Algorithm for 29- to 60-day-old febrile infants.
Figure from Pantell et al. [3]

Ill-appearing infants should always be considered at high risk and therefore undergo blood tests and cultures, hospitalization, lumbar puncture, and prompt empiric antibiotic therapy. In such cases, initial management should focus on supporting the airway, breathing, and circulation. [3,20]

Controversies in the management of febrile infant

Current approaches, protocols, and guidelines for the management of febrile infants leave several controversies and 'gray areas' due to insufficient evidence on which the literature lacks a clear consensus:

- variance in the management of the “middle age group” of infants 22 to 28 days of age [5,9,10,21];
- cut-off age considered at major risk for SBI/IBI at 60 or 90 days[3,8];
- the risk of SBI/IBI in the setting of a positive viral test;
- cut-off of laboratory inflammatory markers;
- how to manage infant with pre-existing risk factors: studies and subsequently protocols and guidelines of febrile infants generally exclude infants with commonly accepted risk factors (Table 4), for which there is a lack of evidence and established management.

Table 4. Inclusion and exclusion criteria for applying the AAP guidelines for febrile infant

Included	May be included	Excluded
Infants 8-60 d of age	Upper respiratory tract infection	Infants < 8 d or > 60 d of age
Temperature ≥ 38°C in past 24h (at home or in clinical setting)	Diarrhea (unless high concern for bacterial pathogen)	Not well-appearing
Term infants ≥ 37 weeks gestation	Recent antibiotic use in infants > 2 weeks of age	High concern for herpes simplex virus
Previously healthy	Positive viral testing	Focal bacterial infection
Well-appearing	Otitis media	Clinical bronchiolitis
		Immunizations in previous 48h
		Medically fragile, chromosomal abnormality
		Known or suspected immunodeficiency
		If < 2 weeks of age and perinatal course with maternal fever or antibiotic use
		Gestational age < 37 weeks

Table modified from [22].

Evidence of viral illnesses

Testing for viral pathogens is evolving, and there are now numerous PCR tests available for a wide array of viruses including rapid point-of-care tests.

Young infants with laboratory-confirmed viral infections are at significantly lower risk for SBI than those in whom no virus has been detected. However, even with a confirmed viral infection, there remains a non-negligible risk of UTI, bacteremia and bacterial meningitis especially under 60 days of age. [23–25]

Moreover, infants ≤90 days with PCR-confirmed rhinovirus infections are more likely to have SBIs than those with other viruses (7.8% versus 3.7%); rhinovirus is also a ubiquitous respiratory virus, often with a protracted shedding period, and frequently presents asymptomatically. [26]

To date, even in the presence of a documented viral pathogen, there is currently no evidence in the literature to support deviations from the current management recommendations for febrile infants.

As suggested by the Canadian position statement, it could be reasonable using a positive finding for any virus (other than rhinovirus) to guide subsequent management decisions (such as withholding or stopping antibiotics, or hospitalizing or discharging otherwise low-risk infants).[20]

1.4 Inflammatory biomarkers

1.4.1 Inflammatory biomarkers commonly used in clinical practice

Since the clinical exam is less reliable in risk stratifying these infants, the majority of current prediction rules and algorithms for the management of febrile infant includes laboratory tests for the evaluation of inflammatory markers (IMs).

The inflammatory markers currently used in the assessment of febrile infants are: white blood cell (WBC) count, absolute neutrophil count (ANC), C-reactive protein (CRP) and procalcitonin (PCT), variably used depending on the protocol.

The peripheral WBC count and ANC have historically been included in most studies attempting to predict the risk of IBI, but they do not perform well in isolation.[22]

The WBC count is not recommended for risk stratification due to its poor performance in screening. The ANC has shown to be a helpful predictive factor in combination with other clinical features or laboratory results. The AAP guidelines include both greater than 4,000/mL (if PCT level is also available) or greater than 5,200/mL (independent of the level of PCT) as cut-off values for abnormal ANC. [3]

Numerous studies have shown that CRP and PCT levels both perform better than WBC count or ANC as predictive measures for SBI and IBI and that PCT level rises more quickly than CRP level and it is the best predictor of the currently available tests. CRP level performs better than ANC or WBC and is likely available at most institutions with a quicker turnaround time for the result and, therefore, may be used when PCT level is not available. [27,28]

These findings were also observed in the population younger than 90 days of age. [29–33]

1.4.2 Novel biomarkers distinguishing between bacterial and viral infections

In recent years, a growing body of evidence have suggested that infections' etiology (viral or bacterial) could be identified by the pattern of host genes activated during the inflammatory response.

Microbial pathogens induce specific host responses or "RNA biosignatures" that can be identified using microarray analyses of blood leukocytes and multiple studies have demonstrated the value of the host's peripheral blood gene expression response signatures to accurately discriminate bacterial, viral, and non- infectious etiologies suggesting that it can represent a diagnostic strategy complementary to those already in use. [34–39]

The use of these diagnostics has not yet been fully defined, especially in children. However, it is known that peripheral blood cells share more than 80% of their transcriptome with those of specific organs; for this reason, whole-blood expression profiling can be used to identify distinct disease signatures regardless of the site of infection.[40] Furthermore, in some studies, blood gene expression turned out to be superior to procalcitonin both with respect to the identification of bacterial infection and the ability to discriminate viral from non-infectious disease. [37,41]

Regarding the population of febrile young infants, Mahajan et al. evaluated the accuracy of RNA biosignatures in febrile infants aged 60 days or younger at their initial evaluation in the ED and defined a 66-transcript blood RNA signature that well discriminated between infants with and without bacterial infections showing that, despite their young age, they carried robust RNA biosignatures and that regardless of the etiology of the infections, their immune systems are programmed to respond with specific patterns that allow discrimination by class of pathogen.[42]

Herberg and colleagues, in a preliminary cross-sectional study of 370 febrile children (aged <17 years), identified a 2-transcript RNA signature (interferon-induced protein 44-like, *IFI44L* and family with sequence similarity 89 member A, *FAM89A*) capable of differentiating between bacterial and viral infections with high sensibility and specificity; this 2-transcript RNA signature showed high sensitivity and specificity also in the specific population of infants younger than 60 days as shown by Kaforou et al.[43] Furthermore Barral-Arca et al. validated this signature using whole transcriptome data from patients suffering from acute diarrhea with bacterial and viral etiology; they found that this signature clearly discriminated between viral and bacterial infections regardless of the pathogen, severity and ancestry of patients.[44]

Type I interferons

Type I interferons (IFNs) are a group of cytokines that are involved in the innate antiviral response and mediate numerous immune interactions during viral infections.[45]

Because IFNs are secreted at very low concentrations (femtomolar) during disease course, directly detecting type I IFN in patients remains a challenge and has led several research groups to explore alternative approaches for monitoring these cytokines. Based on the quantification of expression of subset of genes correlating to the activation of type I IFN, blood transcriptional signatures, referred to as “type I IFN signature” or “IFN signature” provide an indirect estimate of the exposure of cells to type I IFN.[46,47]

Trouillet-Assant et al. found that IFN-alpha concentration and IFN score (mRNA quantification of six IFN-stimulated genes) were significantly higher in viral compared to bacterial infections in a cohort of febrile children aged 7 days to 36 months and that both serum IFN-alpha concentration and IFN score robustly discriminated (Area Under

the Curve AUC >0.91 for both) between viral and bacterial infection compared to C-reactive protein (AUC 0.83) [46].

As previously mentioned, several studies have demonstrated that a 2-transcript host expression signature including IFI44L can accurately discriminate between bacterial and viral etiologies with high sensitivity and specificity in febrile children.[35,43,44,48,49] Furthermore, Gomez-Carballa et al. found that the expression of the IFI44L gene alone not only sufficed to differentiate accurately between viral and bacterial children with fever, but it also performed slightly better than the 2-transcript signature.[35]

Gao et al. found that the IFN I inducible protein 27 (*IFI27*) was highly expressed in preterm RSV-infected infants and its expression correlated with the severity of the disease.[50]

Chawla and colleagues evaluated the performance of 30 published signatures of infection and, in the case of viral infections, they observed that all robust signatures included members of the type I IFN pathway.[51]

Bodkin et al.'s systematic comparison of the published host gene expression signatures for bacterial/viral discrimination showed that two type I interferon-stimulated genes, IFI27 and IFI44L, are commonly used in signature's list and IFI27 in particular turned out to be one of the most important genes for bacterial and viral classification.[52]

Furthermore, McClain et al. found a host gene expression-based assay that accurately predicted a respiratory viral infection before typical symptoms are present, confirming that IFI44L and IFI27 perform well as classifiers to distinguish between individuals with viral illness and those who are healthy.[53]

2.Objectives

To evaluate the ability of peripheral blood IFN signature to early distinguish bacterial from viral infection in febrile infants aged 90 days or younger presenting to the pediatric emergency department.

3.Materials and methods

3.1 Population

This was an observational prospective, single-center, non-profit study.

All infants aged 90 days or younger presented at the Gaslini Hospital's Pediatric Emergency Department (PED) from January 2023 to September 2024 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 hours prior to arrival, were eligible for enrollment. Infants were excluded if they had received antibiotics within 48 hours of PED presentation, if at least one measurement of complete blood cell count (CBC) and 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. Three healthy controls were also included.

3.2 Study design and procedures

Physicians who evaluated the patients in the PED performed a standard history and physical examination on all enrolled patients.

Data collected included patient age, gestational age, gender, medical history, duration of fever. Standard laboratory investigations for febrile infants were performed and a blood sample for interferon signature's analysis was collected. Analysis and coltures of urine,

blood, and cerebrospinal fluid (CSF) was part of the standard evaluation for fever in these infants, however, these investigations were not performed in all cases due to clinical judgment; we included in the study all those who performed at least one determination of CBC and CRP or PCT. Based on the results of laboratory tests (blood, urine, cerebrospinal fluid, nasopharyngeal swabs) and clinical course, subjects were categorized into two groups: those with bacterial infection (BI) and those without bacterial infection (non-BI).

3.2.1 IFN signature and IFN score

Peripheral blood samples (1,5-2,5 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 analysis. After retro-transcription of RNA (up to 1 ug, The SuperScript VILO cDNA Synthesis Kit, Thermo Fisher, USA), cDNAs were stored at -20 °C.

A synthetic oligonucleotide was designed containing the regions of the 8 genes (IFI27, IFI44L, IFIT1, ISG15, RSAD2, SIGLEC1, TBP, HPRT1) recognized by Real-Time PCR primers (gBlocks, IDT, USA) and NotI and XhoI sequence at 5' and 3' respectively. The oligonucleotide was cloned into pCR 2.1 plasmid (Invitrogen, Thermo Fisher, USA) after digestion with NotI and XhoI.

IFN Signature was assessed by calculating the expression of six IFN-stimulated genes (ISGs) (IFI27, IFI44L, IFIT1, ISG15, RSAD2, and SIGLEC1). Real-Time PCR was performed using gene-specific custom-designed FAM probes (TibMolBiol, Roche, Germany) in duplicate. Gene copy number were calculated as follow:

1) standard curves were prepared for each gene with 10-fold serial dilutions of the synthetic control;

2) curves were generated by linear regression analysis, using the function Y = mX + b, where Y is the cycle threshold (Ct), m is the slope of the regression line, calculated as:

$$m = \frac{\sum (x-\bar{x})(y-\bar{y})}{\sum (x-\bar{x})^2}$$

and b is the estimated intercept plotting the Ct value in the Y-axis with the logarithm of the starting RNA dilutions in the X-axis.

Values were normalized with the geometric means of two internal controls (TBP and HPRT1) and expressed as gene score (GS). IFN Score is calculated as the geometric mean of the GS of the six ISGs.

3.3 Study definitions and outcome measures

Bacteremia and bacterial meningitis were defined as the growth of a single pathogen in the blood or cerebrospinal fluid (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: ≥100.000 CFUs/mL in association with an abnormal dipstick test or urinalysis (positive for leukocyte, leukocyte esterase or nitrites).

We considered blood and CSF cultures to reflect growth of contaminants when the bacterial isolates were not commonly accepted pathogens.

We defined SBI as the presence of bacterial meningitis, bacteremia and UTI and IBI as the presence of bacterial meningitis or bacteremia.

Patients were classified as having bacterial infections (group “BI”) 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 on stool samples, depending on the clinical context.

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

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

3.4 Statistical analysis

We compared the demographics, laboratory results and IFN score between BI, non-BI, and HC groups. Data were organized in tabular form and analyzed in an anonymous form. Comparisons between categorical variables were performed using cross-tabulations, and statistical significance was assessed through a 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 among two groups, the choice between parametric (two-sided Student's t-test) and non-parametric (two-sided Mann-Whitney U test) test was based on the data distribution and sample size. A logistic regression model was employed to predict the probability of having a bacterial or non-bacterial infection based on a set of predictor variables. The model's fit was assessed using the area under the

receiver operating characteristic curve (AUC-ROC). All statistical analyses were performed with GraphPad Prism version 9.1.0 for Windows (GraphPad Software, San Diego, California, USA, www.graphpad.com).

4. Results

Study Population

Forty-nine febrile neonates and infants aged 90 days or younger who presented to the PED of Gaslini Children Hospital between January 2023 and September 2024 were enrolled. Two subjects were excluded from subsequent analysis because the RNA sample was not analyzable. Three healthy infants were enrolled as controls (Figure 4).

The median age was 40 days (95% CI 32-47 days) and 40% (n=20) of the subjects were female. Age distribution was as follows: ≤28 days 44% (n=22), 29-60 days 30% (n=15), and 61-90 days 26% (n=13). Of the 47 febrile subjects enrolled, 8 (17%) were assigned to the "Bacterial Infection" (BI) group, while 39 (83%) were assigned to the "Non-Bacterial Infection" (non-BI) group (Figure 4).

There were no significant differences in age and sex between these two groups and the healthy controls (HC) (Table 5).

Table 5. Demographic characteristics

	Overall (n=50)	non-BI (n=39)	BI (n=8)	HC (n=3)	P value
Age (days) **(Mean, CI 95%)**	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%)	

Bacterial Infections

Eight infants (17%) had a bacterial infection, of these, 5 had IBIs with isolated bacteremia, and 3 had UTI without bacteremia. No cases of bacterial meningitis were observed. IBIs were identified in 2 of 22 infants aged 28 days and younger (9%), in 3 of 15 infants aged 29 to 60 days (20%) and none in infants aged 61 to 90 days. Bacterial infection type and pathogens are listed in Table 6.

Table 6. Bacterial infection type and pathogens stratified by age group

	Bacterial infection		
Age (days)	**Bacteremia**	**Meningitis**	**UTI**
0-28	2 *MSSA* *Streptococcus salivarius*	0	1 *Escherichia coli*
29-60	3 *Streptococcus agalactiae* *Staphylococcus epidermidis* *Enterobacter cloacae*	0	0
61-90	0	0	2 *Streptococcus agalactiae* *Escherichia coli*

MSSA: meticillin-sensitive Staphylococcus aureus; UTI: urinary tract infection.

Non-bacterial infections

Thirty-nine infants (83%) were classified as non-bacterial infection. Of these, 19 had no identified pathogen. In 19 a viral pathogen was detected between *parechovirus, enterovirus, adenovirus, metapneumovirus, parainfluenza virus, SARS-CoV-2 virus*, and *respiratory syncytial virus* (*RSV)* (Table 7); a single patient was diagnosed with a *Candida glabrata* urinary tract infection.

Table 7. Viral pathogens observed and site of isolation

Virus	N	Site of virus isolation
Parechovirus	7	Liquor
Enterovirus	3	Liquor
Enterovirus and Adenovirus	1	Liquor and Nasopharyngeal swab
RSV	1	Nasopharyngeal swab
Rhinovirus	2	Nasopharyngeal swab
Metapneumovirus and Parainfluenzae virus	1	Nasopharyngeal swab
Sars-Cov2 virus	4	Nasopharyngeal swab

RSV: respiratory syncytial virus.

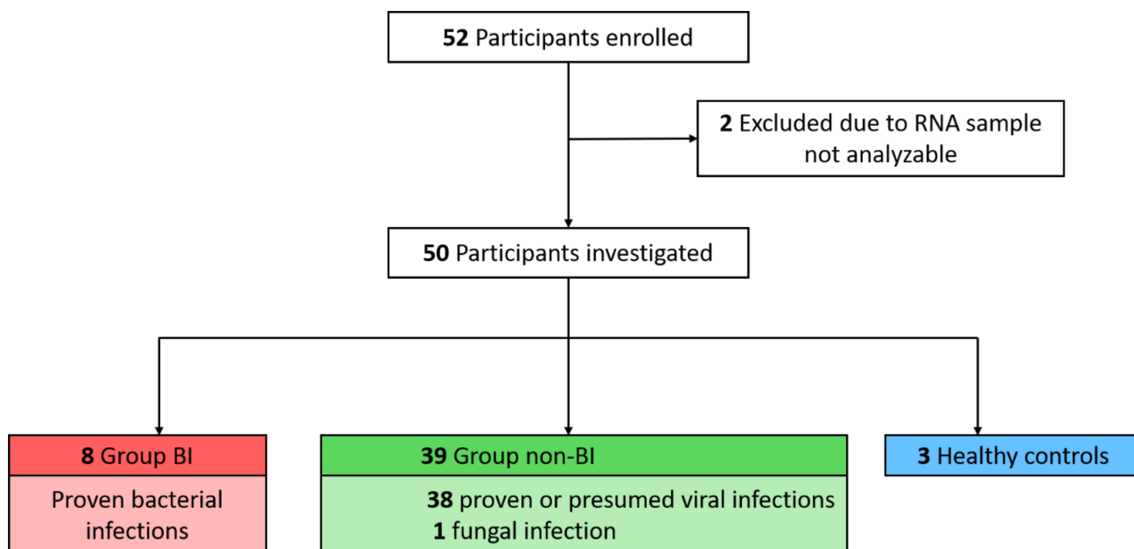

Figure 4. Flow diagram of study population.

Inflammatory markers

CRP levels resulted significantly higher in the BI group compared to both the non-BI (p=0.002) and HC groups (p=0.02); PCT levels were also significantly higher in the BI group compared to the non-BI group (p<0.001). PCT wasn't measured in the healthy control group. Analysis of the leukocyte count revealed a significant increase of WBC in the BI group compared to non-BI (p=0.02). ANC resulted significantly higher in the BI group compared to both non-BI and HC groups (p=0.03 and p=0.03, respectively). Interestingly, the absolute lymphocyte count (ALC) was significantly decreased in both BI and non-BI groups compared to HC (p=0.04 and p=0.04, respectively) (Figure 5 and Table 8).

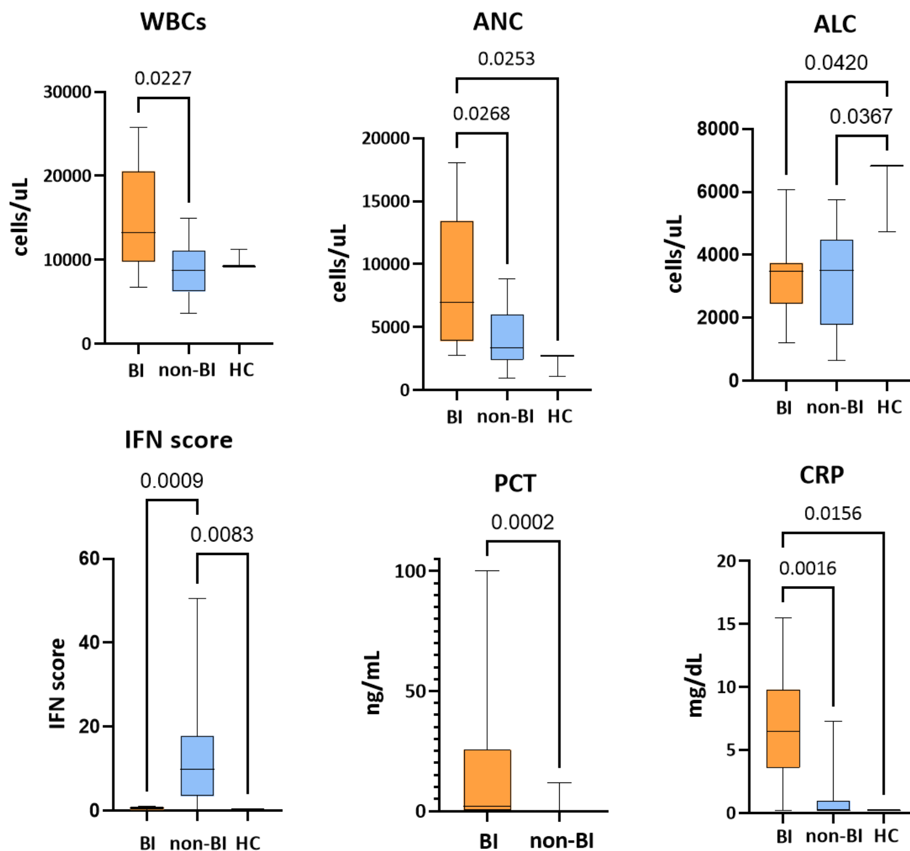

Figure 5. Comparison of inflammatory markers between BI, non-BI, and HC. The box bounds the IQR divided by the median, and the whiskers extend to the 5th and 95th percentiles. P values were assessed through Dunn's test and adjusted using the Benjamini-Hochberg correction for multiple tests (p value for PCT was instead calculated using Mann-Whitney U test). BI: bacterial infections group; non-BI: non-bacterial infections group; HC: healthy controls.

IFN score

IFN score differed significantly among the three groups (p < 0.0001). Specifically, IFN score resulted elevated in non-BI group compared to both the BI (p< 0,001) and healthy control groups (p=0.008). No significant difference was observed between the healthy control and BI groups (Figure 5 and Table 8).

Table 8. Comparison of traditional inflammatory markers and IFN score between group BI, non-BI and HC.

	BI (A)	non-BI (B)	HC (C)	P value	Adj P value
CRP mg/dL Median (IQR)	6.5 (3.6-9.8)	0.23 (0.23-0.98)	0.23 (0.23-0.23)	0.001	A-B 0.002 A-C 0.02
PCT ng/mL Median (IQR)	1.9 (0.5-25.6)	0.1 (0.1-0.3)	NA	< 0,001*	
WBC cells/µL Median (IQR)	13265 (9783-20555)	8760 (6210-11110)	9240 (9160-11270)	0.02	A-B 0.02
ANC cells/µL Median (IQR)	6960 (3890-13460)	3390 (2410-6020)	2740 (1060-2740)	0.02	A-B 0.03 A-C 0.03
ALC cells/µL Median (IQR)	3480 (2450-3750)	3500 (1780-4480)	6830 (4730-6840)	0.04	A-C 0.04 B-C 0.04
IFN score Median (IQR)	0.67 (0.24-0.92)	9.86 (3.40-17.84)	0.33 (0.31-0.33)	p<0.0001	A-B <0.001 B-C 0.008

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.
*Mann-Whitney test between A and B
ANC: absolute neutrophil count; ALC: absolute lymphocyte count (ALC); IQR: interquartile range; WBC: white blood cell count.

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/risk factors are at increased risk of SBI. A comprehensive list of the identified comorbidities is provided in Table 9.

Table 9. Comorbidities observed in our cohort

Comorbidity	Infection observed
Empirical antibiotic therapy at birth due to maternal fever (maternal vaginal-rectal swabs negative, no bacterial isolation from patient)	-Enterovirus
Maternal vaginal and/or rectal swabs positive for GBS and either no or incomplete antenatal prophylaxis	-Rhinovirus -UTI Escherichia Coli
Unilateral pyelectasis	-UTI Escherichia Coli -Unknown pathogen
History of GBS sepsis	-GBS sepsis
History of febrile episodes treated with antibiotics	-S. epidermidis sepsis
Recent surgery	-Enterobacter Cloacae sepsis
Prematurity	-UTI GBS
Patient under investigation for suspected megacolon	-UTI Escherichia Coli
Multiple congenital anomalies consistent with the VACTERL association*	-UTI Candida Glabrata

GBS: Group B Streptococcus; UTI: urinary tract infection.
*: surgically corrected type III esophageal atresia, colostomy for anorectal malformation, chronic kidney disease and bilateral hydronephrosis for vesicoureteral reflux.

In the cohort of patients with comorbidities who tested negative for bacterial infections, one had a fungal (candida glabrata) UTI, and three had confirmed or presumed viral infections, of these, two received empirical antibiotic therapy.

When comparing CRP, PCT, WBC, ANC, and IFN score values between patients with bacterial infection (n=8) and those with viral infection and pre-existing comorbidities (n=3), PCT and IFN score 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 score in predicting the presence of bacterial infection, 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) (Figure 6).

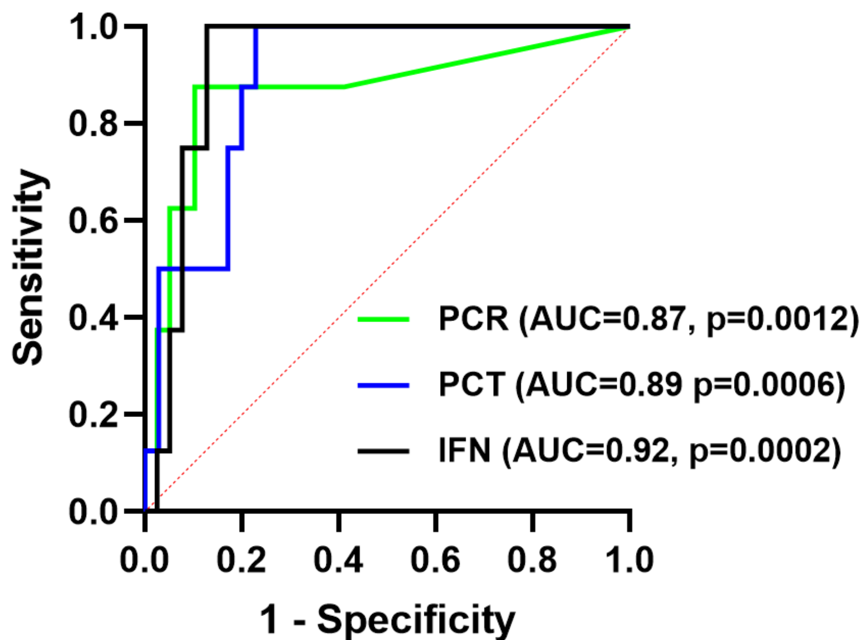

Figure 6. ROC curve analysis of inflammatory markers for bacterial infection prediction.
AUC: area under the curve.

When analyzing the performance of markers in pairs, models utilizing IFN score in combination with PCT or CRP showed superior performance compared to models using only CRP and PCT. Specifically, the IFN+PCR 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 (Figure 7).

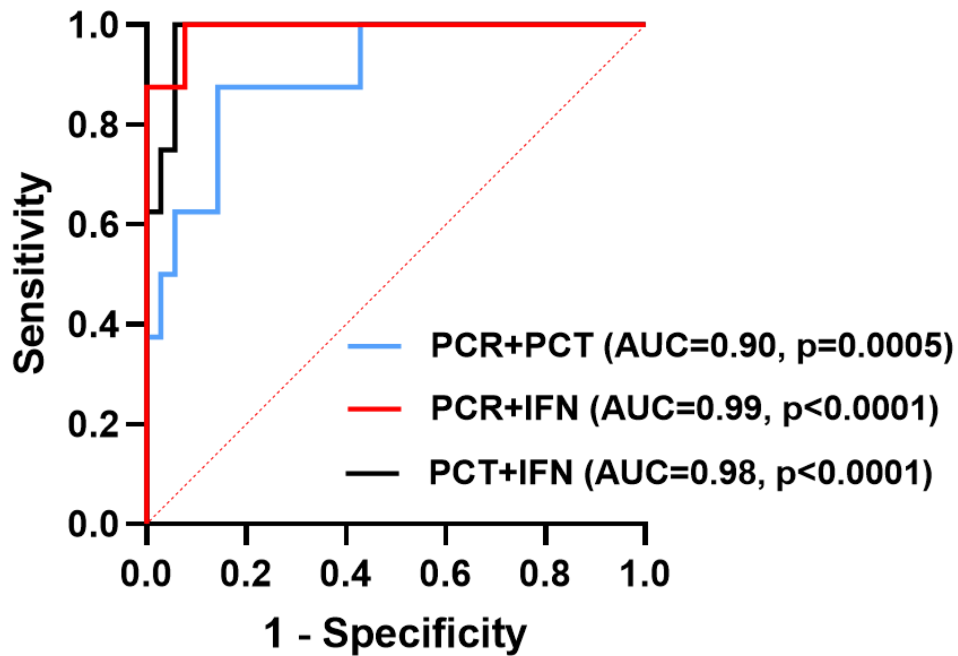

Figure 7. ROC curve analysis of inflammatory markers in pairs for bacterial infection prediction.
AUC: area under the curve.

Inflammatory Markers and Duration of Fever

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

When analyzing data obtained **within the initial 12 hours of fever onset**, significant differences were observed in 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 (Table 10 and figure 8).

Table 10. Markers performed within the first 12 hours of fever

	BI (n=3)	non-BI (n=26)§	p Value
< 12h of fever onset			
IFN score Median (IQR)	0.7 (0.2-1.0)	10.6 (3.4-29.4)	0.03*
CRP mg/dL Median (IQR)	3.5 (0.2-8.1)	0.2 (0.2-0.5)	0.04*
PCT ng/mL Median (IQR)	0.71 (0.47-100)	0.14 (0.09-0.27)	0.02*
WBC cells/µL Median (IQR)	14640 (10120-20700)	8225 (4735-10990)	0.03*
ANC cells/µL Median (IQR)	9750 (6290-18040)	3340 (3340-5170)	0.006**
ALC cells/µL Median (IQR)	2450 (1200-3480)	2535 (1555-3953)	0.66

CRP: C-reactive protein; PCT: procalcitonin; ANC: absolute neutrophil count; ALC: absolute lymphocyte count; IQR: interquartile range; WBC: white blood cell count.
§ =only 21 samples were collected for IFN score detection in non-BI individuals within 12 hours of fever.

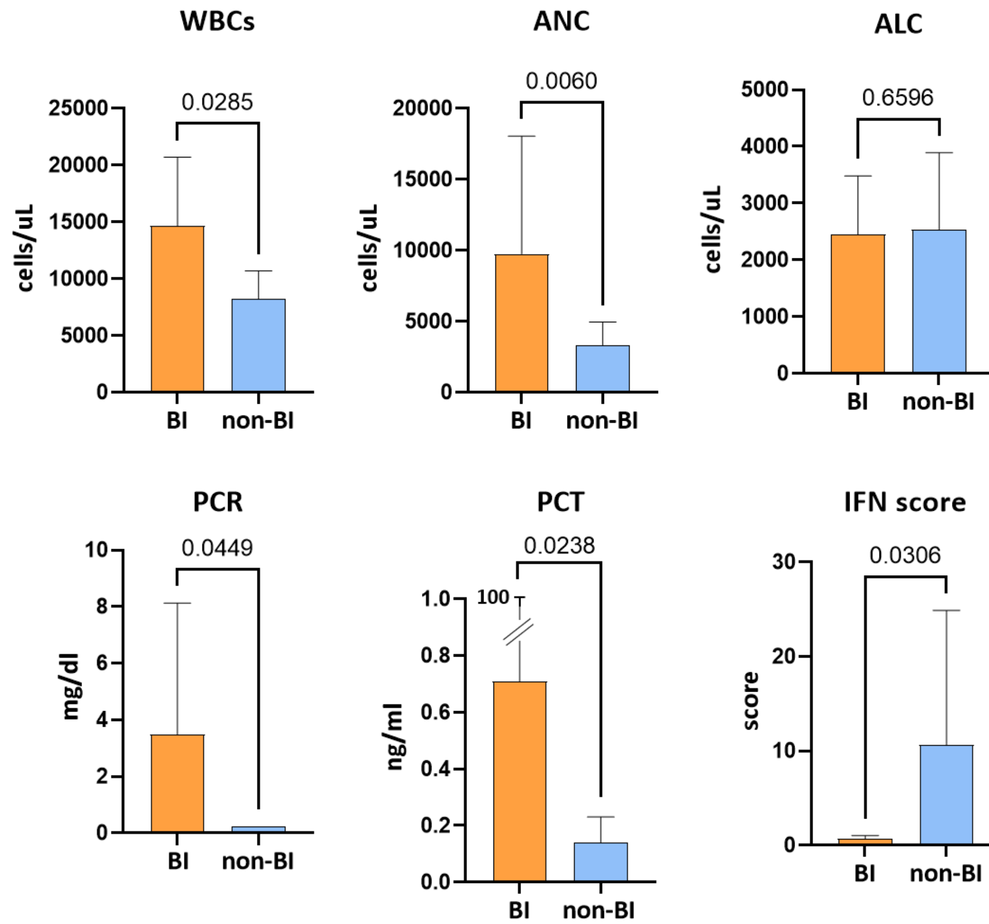

Figure 8. Comparison of inflammatory markers and IFN score performed within 12h from fever onset between BI and non-BI groups.

The histograms show the medians with 95% CI. P values are based on Mann-Whitney U test. BI: bacterial infections group; non-BI: non-bacterial infections group.

Viral infections and IFN score

We conducted a comparison of IFN scores among the different viruses identified in our sample to assess whether there were variations in IFN pathway induction and no significant differences were found (Figure 9).

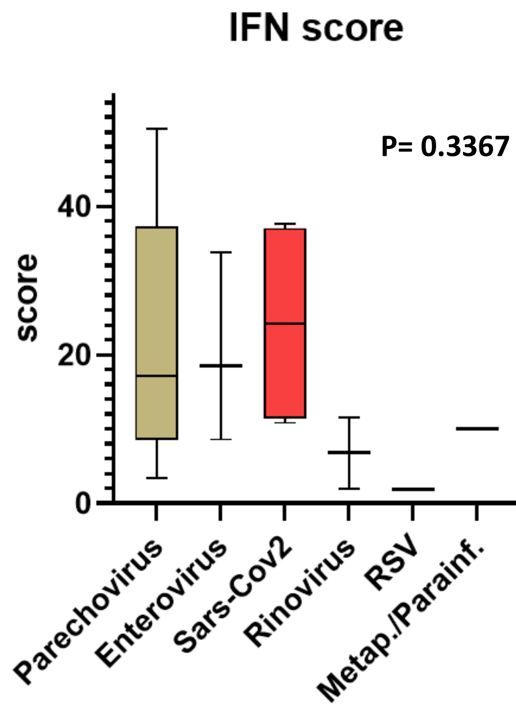

Figure 9. Comparison of IFN score among viral etiologies.
P value was calculated using Kruskal-Wallis test. RSV: respiratory syncytial virus; Metap.: metapneumovirus; Parainf.: parainfluenza virus.

Predictive probability of bacterial infection of different logistic regression models

We subsequently analyzed the cohort of subjects who eventually tested negative for bacterial infection (non-BI) but had received empirical antibiotic therapy. Within the non-BI group, out of 39 subjects, 14 (36%) received empirical antibiotic therapy. Of these, 5 resulted positive for Parechovirus, 3 for Enterovirus, 1 for both enterovirus and adenovirus, 1 for RSV, 1 for Rhinovirus, and for 3 the pathogen was not identified. Applying the logistic regression models previously analyzed to this cohort, the predictive probability (PP) of bacterial infection (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 PCR+IFN score model, the PP of BI improved further with extremely low values (≤ 0.3%) for 13/14 subjects. The CRP+PCT model was found to be the least performant in identifying bacterial infections (Table 11). The mean age of this cohort was 17 days, and 12 out of 14 were less than 28 days old.

Table 11. Predictive probability of BI of different logistic regression models

Subject	Age (days)	Etiological agent	CRP+PCT	IFN score	PCR+IFN score
1	3	*NA*	5,4%	23.0%	0.3%
2	6	Parechovirus	5.4%	< 0,001%	< 0,001%
3	9	Enterovirus	5,3%	< 0,001%	< 0,001%
4	11	Rinovirus	21.8%	12.4%	0.3%
5	11	*NA*	34.6%	< 0,001%	< 0,001%
6	12	RSV	73.4%	13.1%	3.5%
7	12	Enterovirus+adenovirus	7.7%	< 0,001%	< 0,001%
8	14	Enterovirus	34.8%	< 0,001%	< 0,001%
9	16	Parechovirus	5.3%	1.4%	< 0,001%
10	20	Parechovirus	5.9%	< 0,001%	< 0,001%
11	22	Parechovirus	6.0%	< 0,001%	< 0,001%
12	22	*NA*	5.3%	0.1%	< 0,001%
13	38	Enterovirus	5.8%	0.1%	< 0,001%
14	44	Parechovirus	NA	< 0,001%	< 0,001%

5. Discussion

To our knowledge, this is the first study to analyze the type I IFN signature in a population of 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.[42–44,48]

Our data demonstrate that the IFN score is significantly higher in non-bacterial infections, while no differences in its value were found between the BI group and healthy controls. These findings align with previous literature, which has consistently shown that the interferon pathway is activated during viral infections but not during bacterial ones.[35,43,44,46,48,49]

In our logistic regression models, the interferon signature alone demonstrated greater sensitivity and specificity than the combination of CRP and PCT in discriminating between bacterial and non-bacterial infections. Furthermore, the combined use of the interferon signature and PCT or CRP resulted in an even higher performance (AUC 0.98 and 0.99 respectively). Similar findings have been previously reported in the literature for populations older than our cohort.[46]

In our analysis of non-BI patients who received empirical antibiotic therapy, we observed that our IFN score model yielded exceptionally low predictive probability for bacterial infections (<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 could 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 population, they should be validated in an independent cohort.

Current available inflammatory markers, combined with clinical evaluation, are insufficiently sensitive and specific to early discriminate SBI in this febrile young population[3–7] and culture of bacteria from normally sterile sites, which are currently the gold standard for confirming an ongoing bacterial infection, take several days for results, could be negative when 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's immune response could provide an essential contribution to the early identification of severe bacterial infections. Our study demonstrates that the IFN score alone is capable of discriminating between bacterial and non-bacterial infections, and when used as a complement to current markers, it further increases both sensitivity and specificity of the tests.

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.[9,10,54] Our findings are consistent with these conclusions, indeed, in our cohort, no inflammatory markers assessed within the first 6 hours 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 hours (CRP, PCT, WBC, and ANC). We suggest the need to monitor patients with normal blood tests but fever onset less than 6 hours, repeating inflammatory markers after this time.

Due to insufficient data, we were unable to assess the IFN score within the first 6 hours of fever. However, the test demonstrated reliability when performed within the first 12

hours 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[45] and that there are evidences that host gene expression can detect a viral infection before the onset of syntoms.[53]

Limitations

This study has some important limitations: a small sample size and the absence of an independent validation dataset, a limited number of healthy controls, a lack of data on IFN score in cases of bacterial and viral co-infection and lack of in identification of the causative agent in many non-bacterial infections.

6. Conclusion

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

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

Further prospective research with larger populations is needed to refine and validate the estimates of test accuracy and to assess the clinical utility of IFN score in practice.

Additionally, research should investigate the feasibility of implementing IFN score testing using clinical assays suitable for hospital laboratories or point-of-care settings.

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