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Potential of Myxovirus Resistance Protein A

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To my Mom,
who gave me the colour of my eyes, and everything I ever needed in life,
and to Jani,
without whom this book would have taken even longer to complete

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ABSTRACT

Differentiating between viral and bacterial infections in children is a diagnostic challenge, often leading to antibiotic overuse and driving antimicrobial resistance. Current diagnostic methods have notable limitations, highlighting the need for novel tools. Blood myxovirus resistance protein A (MxA) is a promising biomarker for viral infections, given its broad antiviral activity and rapid induction during viral illness. Host gene expression analysis further expands diagnostic possibilities by identifying biosignatures unique to viral or bacterial infections.

This thesis comprises four studies. Study I assessed respiratory viruses and antiviral MxA responses in children with febrile urinary tract infections (UTIs). Study II evaluated MxA as a biomarker for viral infection in children hospitalised with a suspected serious infection. Study III investigated the accuracy of a novel point-of-care (POC) MxA test and its ability to differentiate viral from bacterial infections in febrile children at the emergency department. Study IV explored host gene expression signatures to distinguish bacterial from viral infections.

In this study, respiratory viruses were frequently detected in children with febrile UTIs and other serious bacterial infections. Blood MxA levels were significantly higher in viral infections and viral-bacterial coinfections compared to bacterial infections. In children with a suspected serious infection, a blood MxA cutoff of 256 µg/L differentiated viral from bacterial infections with a sensitivity of 74% and specificity of 80%. The novel POC MxA test provided rapid results with acceptable accuracy compared to the reference method, supporting its use in acute care settings. A POC MxA level of 101 µg/L differentiated between viral and bacterial infections with 92% sensitivity and 91% specificity. A 2-transcript host gene expression signature (*TSPO* and *SECISBP2*) distinguished bacterial and viral-bacterial coinfections from viral infections with 77% sensitivity and 87% specificity.

This thesis highlights MxA and host gene expression analysis as promising advancements in paediatric infectious disease diagnostics. The high prevalence of viral-bacterial coinfections challenges novel diagnostic approaches and supports the use of MxA in combination with bacterial biomarkers.

KEYWORDS: viral infection, bacterial infection, respiratory tract infection, children, host response, myxovirus resistance protein A (MxA), gene expression, point-of-care (POC) test

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TIIVISTELMÄ

Lasten virus- ja bakteeri-infektioiden erotusdiagnostiikkaan tarvitaan uusia menetelmiä, sillä nykyinen diagnostinen epävarmuus johtaa antibioottien liikakäyttöön ja pahentaa antibioottiresistenssiä. Myksovirusresistenssiproteiini A (MxA) on lupaava virusinfektion merkkiaine, sillä sen pitoisuus veressä suurenee nopeasti useimpien virusinfektioiden yhteydessä. Veren geenien ekspressioanalyysin avulla voidaan määrittää virus- ja bakteeri-infektioille ominaisia muutoksia geenien ilmentymisessä.

Väitöskirja koostuu neljästä tutkimuksesta. Ensimmäisessä osatyössä tutkimme hengitystievirusten esiintymistä ja antiviraalista MxA-vastetta kuumeista virtsatieinfektiota sairastavilla lapsilla. Toisessa osatyössä arvioimme MxA:n suorituskykyä virusinfektion merkkiaineena sairaalahoitoon vakavan bakteeri-infektion epäilyn vuoksi otetuilla lapsilla. Kolmannessa osatyössä vertasimme uuden MxA-pikatestin mittaustarkkuutta laboratoriomenetelmään verrattuna. Arvioimme myös MxA:n kykyä erottaa kuumeisten lasten virus- ja bakteeri-infektiot toisistaan päivystyspoliklinikalla. Neljännessä osatyössä tutkimme geenien ekspressioanalyysin potentiaalia virus- ja bakteeri-infektioiden erotusdiagnostiikassa.

Tutkimuksessa kuumeiseen virtsatieinfektioon ja muihin bakteeri-infektioihin sairastuneilta lapsilta löydettiin usein hengitystieviruksia. Veren MxA-pitoisuus oli selvästi suurempi virusinfektiota tai viruksen ja bakteerin sekainfektiota sairastavilla lapsilla verrattuna yksinomaan bakteeritautia sairastaviin. Sairaalahoitoisilla potilailla MxA:n raja-arvo 256 µg/l erotteli virusinfektiot bakteeritaudeista 74 %:n herkkyydellä ja 80 %:n tarkkuudella. MxA-pikatestin todettiin soveltuvan päivystyskäyttöön, sillä sen suorittaminen oli nopeaa ja mittaustarkkuus riittävä. Pikatestin raja-arvolla 101 µg/l eroteltiin virus- ja bakteeri-infektiot 92 %:n herkkyydellä ja 91 %:n tarkkuudella. Geenien ekspressioanalyysissä kahden eri tavoin ilmentyvän geenin yhdistelmä (*TSPO* ja *SECISBP2*) erotti bakteeri- ja sekainfektiot virustaudeista 77 %:n herkkyydellä ja 87 %:n tarkkuudella.

MxA-määritys ja geenien ekspressioanalyysi ovat lupaavia menetelmiä lasten infektiosairauksien diagnostiikassa. Sekainfektioiden yleisyyden vuoksi MxA-määritys kannattaa yhdistää bakteeri-infektion merkkiaineen määritykseen.

AVAINSANAT: virusinfektio, bakteeri-infektio, hengitystieinfektio, lapset, tulehdusvaste, myksovirusresistenssiproteiini A (MxA), geenien ekspressio, pikatesti

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Abbreviations

AI	artificial intelligence
AMR	antimicrobial resistance
ANC	absolute neutrophil count
AUC	area under the curve
BSE	bundle signalling element
CI	confidence interval
CRP	C-reactive protein
ED	emergency department
EIA	enzyme immunoassay
GTP/GTPase	guanosine triphosphate/guanosine triphosphatase
IBI	invasive bacterial infection
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ML	machine learning
mNGS	metagenomic next generation sequencing
MxA	myxovirus resistance protein A
NAAT	nucleic acid amplification test
PCR/qPCR	polymerase chain reaction/quantitative polymerase chain reaction
PCT	procalcitonin
POC	point-of-care
SARS-CoV	severe acute respiratory syndrome coronavirus
SBI	serious bacterial infection
RNA-seq	RNA sequencing
ROC	receiver operating characteristic
RSV	respiratory syncytial virus
RTI	respiratory tract infection
UTI	urinary tract infection
TRAIL	tumour necrosis factor-related apoptosis-inducing ligand
WBC	white blood cell count
WHO	World Health Organization

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Piri R, Ivaska L, Yahya M, Toivonen L, Lempainen J, Kataja J, Nuolivirta K, Tripathi L, Waris M, Peltola V. Prevalence of respiratory viruses and antiviral MxA responses in children with febrile urinary tract infection. *Eur J Clin Microbiol Infect Dis.* 2020;39(7):1239-1244

II Piri R, Yahya M, Ivaska L, Toivonen L, Lempainen J, Nuolivirta K, Tripathi L, Waris M, Peltola V. Myxovirus Resistance Protein A as a Marker of Viral Cause of Illness in Children Hospitalized with an Acute Infection. *Microbiol Spectr.* 2022;10(1): e0203121

III Piri R, Ivaska L, Kujari AM, Julkunen I, Peltola V, Waris M. Evaluation of a Novel Point-of-Care Blood Myxovirus Resistance Protein A Measurement for the Detection of Viral Infection at the Pediatric Emergency Department. *J Infect Dis.* 2024;230(5):e1049-e1057

IV Piri R, Valta M, Lempainen J, Ivaska L, Ezer S, Kere J, Katayama S, Peltola V. Host Gene Expression Analysis in the Detection of Bacterial and Viral Etiology in Children Hospitalized with a Suspected Severe Infection. *Manuscript*

1 Introduction

Acute infectious diseases remain the leading cause of morbidity and mortality in children worldwide. In paediatric emergency departments (EDs), infections account for up to half of visits, with viral respiratory tract infections being the most common (Sands et al., 2012). Differentiation between viral and bacterial infections is challenging due to overlapping clinical features. While serious bacterial infections now represent a minority of cases in febrile children – largely due to successful vaccination programmes – prompt identification and treatment remain crucial to prevent morbidity and mortality. Diagnostic uncertainty often leads to antibiotic overuse, which not only drives antimicrobial resistance but is also linked to risks such as autoimmune diseases and obesity (Kronman et al., 2012; Saari et al., 2015).

Current diagnostic methods, such as blood bacterial culture, are limited by delayed results and low sensitivity. The frequent detection of respiratory viruses by nucleic acid testing in asymptomatic children complicates the interpretation of viral diagnostic assays. Bacterial biomarkers, such as C-reactive protein and procalcitonin, lack the specificity required to efficiently guide antibiotic use.

Novel diagnostic tools focus on the host immune response rather than direct pathogen detection. Myxovirus resistance protein A (MxA), an interferon-induced protein with antiviral properties, is rapidly elevated during acute viral infections while remaining low in asymptomatic virus-positive children, and those with bacterial infections, making it a promising viral biomarker (Engelmann et al., 2015; Ronni et al., 1993; Toivonen et al., 2015). For MxA to impact diagnostic decision-making, its measurement must be available through a rapid point-of-care (POC) test. Host gene expression profiling further expands diagnostic possibilities by identifying biosignatures unique to viral or bacterial infections (Herberg et al., 2016).

This study evaluated the diagnostic utility of MxA in distinguishing between viral and bacterial infections in children hospitalised for a suspected serious infection and febrile children presenting at the ED. It also explored whether these infections could be differentiated through host gene expression analysis. Furthermore, the study assessed the accuracy and feasibility of a novel POC test for MxA in the paediatric ED. This work aimed to advance paediatric infectious disease diagnostics, with the ultimate goal of improving patient care and addressing antimicrobial resistance.

2 Review of the Literature

2.1 Acute Infections in Children

2.1.1 Epidemiology

Despite major advances in the development of vaccines, monoclonal antibodies, and antimicrobials, acute infectious diseases remain the leading cause of childhood morbidity and mortality worldwide (Naghavi et al., 2017). The incidence and type of infections vary based on factors such as age, comorbidities, vaccination coverage, geography, and season. They contribute to up to half of paediatric ED visits, with viral respiratory tract infections (RTIs) being by far the most common type of illness (Alpern et al., 2006; Sands et al., 2012).

Young children experience an average of six RTIs annually, which drive substantial numbers of antibiotic prescriptions, hospital admissions, and healthcare costs. The highest disease burden is seen in children aged 1 to 2 years (Byington et al., 2015; Jules et al., 2015; Nair et al., 2010; Toivonen et al., 2016). During the 21st century, vaccinations for *Streptococcus pneumoniae* (pneumococcus), influenza and rotavirus have reduced hospitalisations from these pathogens, and the recently introduced monoclonal antibody nirsevimab for respiratory syncytial virus (RSV) shows equally promising outcomes (Assad et al., 2024; Boddington et al., 2021; Luca et al., 2018; Vesikari et al., 2006).

In addition to causing acute infections, certain respiratory viruses, such as rhinoviruses, coronaviruses and human bocavirus, are frequently detected in asymptomatic children. On average, young children are respiratory virus-positive every other week, but only half of these episodes are associated with symptoms. Occasionally, viral detection can persist for more than 3 weeks regardless of symptoms (Byington et al., 2015; Jartti et al., 2008; Rhedin et al., 2014).

While most febrile children at the ED have self-limiting viral illnesses, 7 to 16% will have a serious bacterial infection (SBI) (Craig et al., 2010; Hsiao et al., 2006; Nijman et al., 2014; Pratt & Attia, 2007). SBIs can lead to significant morbidity and mortality if not promptly diagnosed and managed with antibiotics. This term strictly includes invasive bacterial infections (IBIs) – bacteraemic infections and bacterial culture-positive meningitis – as well as urinary tract infections (UTIs). These often

present with fever without localised symptoms, making clinical diagnosis challenging. Although pneumonia accounts for 14% of all deaths among children under 5 years of age globally (World Health Organization, 2022), it is typically considered separately from other SBIs due to the high prevalence of viral causes, especially when using the WHO's broad clinical criteria for pneumonia. Other focal bacterial infections, such as skin and soft tissue infections, are generally easier to diagnose clinically.

The epidemiology of SBIs in children has evolved since the introduction of pneumococcal conjugate vaccines in 2000. Rates of pneumonia and sepsis have declined, making febrile UTI the most common SBI, representing over 80% of cases in infants, and over 90% when invasive UTIs are included (Greenhow et al., 2014; Herz et al., 2006; Shaikh et al., 2008). During the COVID-19 pandemic lockdowns, the absolute number of young febrile infants brought to the EDs decreased substantially; however, the proportion of those with SBIs doubled and those with IBIs tripled (Burstein et al. 2021).

The overall prevalence of UTI in children presenting with fever alone is 2 to 8%, though factors such as age, gender, race, and circumcision status significantly influence the risk (Craig et al., 2010; Hsiao et al., 2006; Nijman et al., 2014). For instance, the likelihood of UTI is as high as 16 to 20% in a febrile white girl under 2 years old or an uncircumcised boy under 3 months old (Shaikh et al., 2008; Shaw et al., 1998). In infants, diagnosing a UTI requires a high level of clinical suspicion, as symptoms, such as fever, irritability, and poor feeding are non-specific. The localising symptoms commonly seen in older children, such as abdominal pain or dysuria, are absent in this age group (National Institute for Health and Care Excellence (NICE), 2022). Although SBI occasionally occurs simultaneously with an RTI, its likelihood is significantly lower among respiratory virus-positive febrile infants than in virus-negative ones (Byington et al., 2004; Mahajan et al., 2018). For instance, in febrile infants younger than 60 days, the probability of a UTI is 3% in virus-positive infants, compared to 11% in virus-negative infants (Mahajan et al., 2018).

Febrile infants aged < 90 days of age are at particularly high risk for bacterial infection and are often considered separately from older children in treatment guidelines (Cioffredi & Jhaveri, 2016; Graaf et al., 2023; Leazer, 2023; National Institute for Health and Care Excellence (NICE), 2021). While these guidelines vary in their specifics, they quite consistently recommend routine biomarker measurement in all febrile infants < 90 days of age. Blood culture and urine analysis are widely endorsed, particularly for neonates < 30 days of age. According to the most recent American Academy of Pediatrics guideline, lumbar puncture is not routinely required in well-appearing febrile infants > 21 days of age with normal inflammatory markers (Leazer, 2023). Also, some guidelines recommend polymerase chain reaction (PCR) testing for viral pathogens to help refine diagnostic decisions. For infants < 30 days of age, antibiotic treatment is almost universally

recommended, regardless of clinical appearance or biomarker findings. In contrast, infants aged 30–90 days are managed with greater clinical discretion, and from 3 months of age, antibiotic treatment is reserved for cases with a high risk of serious infection based on clinical and diagnostic findings (Graaf et al., 2023).

2.1.2 Microbiological Aetiology

Acute infections in paediatric EDs are caused by a broad range of pathogens, primarily viruses. Viruses can be detected in up to 90% of RTIs in children with rhinovirus being the most frequently identified (Martin et al., 2013; A. C. Nascimento-Carvalho et al., 2018; Pöyry et al., 2021; Ruohola et al., 2009). Over 160 rhinovirus types have been described, classified into three species: A, B and the more recently discovered C. Other clinically relevant respiratory viruses include RSV, influenza viruses, parainfluenza viruses, adenovirus, enterovirus, coronaviruses, human bocavirus and human metapneumovirus (Perez et al., 2022; Tregoning & Schwarze, 2010). Important bacterial pathogens – either as primary agents or complicating concurrent viral illness – include pneumococcus, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus pyogenes* (Jain et al., 2015; Ruohola et al., 2006). The first three bacteria colonise the nasopharynx from early infancy and are considered part of the normal flora as well (Faden et al., 1997).

In addition to respiratory infections, viruses are responsible for most childhood enteric infections (e.g., rotavirus, and norovirus) and febrile illnesses without an apparent source (e.g., adenovirus, enterovirus, and herpesviruses) (Colvin et al., 2012; Nicholson et al., 2016; Xie et al., 2019). In previously healthy children with SBI, the most common pathogens include *Escherichia coli*, *Staphylococcus aureus*, and various *Streptococcus* species (Greenhow et al., 2014; Martinón-Torres et al., 2018; Prout et al., 2020).

The simultaneous detection of multiple viruses is common in children, occurring in approximately one-third of RTIs (Aberle et al., 2005; Chonmaitree et al., 2015; Cilla et al., 2008; Honkinen et al., 2012; Jartti et al., 2013; Ruuskanen et al., 2011; Tsolia et al., 2004). However, there is no consensus on the impact of viral-viral coinfection on disease severity. Some studies have reported worse clinical outcomes in cases of multiple compared to single-virus infections, while others have found no such association (Goka et al., 2014; C. M. Nascimento-Carvalho & Ruuskanen, 2016).

Coinfections involving both viruses and bacteria are also common in RTIs (Brealey et al., 2015; O'Grady et al., 2016). As observed with RSV and influenza, viral infections increase the susceptibility to bacterial coinfections, such as acute otitis media or pneumonia, by damaging physical barriers and altering the immune response (Marom et al., 2012; Morens et al., 2008; Peltola et al., 2005; Tregoning & Schwarze, 2010). It has been hypothesised that the interaction between respiratory

viruses and bacteria may be bidirectional, with bacteria potentially influencing host susceptibility to viral infections as well (Brealey et al., 2015). Viral-bacterial codetection is associated with greater disease severity compared to infections caused by either pathogen alone (Brealey et al., 2015, 2018; Marom et al., 2012).

2.1.3 Diagnostic Challenges and Antimicrobial Usage

The differential diagnosis of viral and bacterial infections is complicated by their overlapping symptoms and signs. Timely administered antibiotic treatment is crucial in cases of SBI, particularly in sepsis, where mortality increases by 4–7% for every hour of delayed antibiotics (Rhodes et al., 2017; Seymour et al., 2017; Weiss et al., 2014). In young infants, clinical clues of an SBI can be subtle, if present at all in the early phase (Hilarius et al., 2020; Rose, 2021). Even in older children, warning signs associated with a high positive likelihood ratio of an SBI might not raise the probability of the disease above 5% in primary care settings (Van Den Bruel et al., 2010). Current diagnostic methods have several limitations. For instance, in a multicentre European study on children hospitalised with sepsis or severe focal infections, a causative pathogen was identified in less than 50% of cases (Martinón-Torres et al., 2018). The frequent detection of respiratory viruses in asymptomatic children further complicates the interpretation of diagnostic tests (Jansen et al., 2011; Jartti et al., 2008; Rhedin et al., 2014). No biomarker for viral infection is yet in routine clinical use.

With the incidence of SBIs declining over time, antibiotic treatment is often unnecessary and potentially harmful for most febrile children presenting to the ED. In high-income countries, an estimated 4 to 33% of children with viral infections receive unnecessary antibiotics, reflecting both the diagnostic uncertainty and concern of missing a bacterial infection (Desai et al., 2020; Fitzpatrick et al., 2021; Fleming-Dutra et al., 2016; Hagedoorn et al., 2020; Hersh et al., 2011; Korppi et al., 2022; S. Shah et al., 2016). Antibiotic prescription rates are highest among children under 2 years of age due to the high disease burden in this age group (Fleming-Dutra et al., 2016; Rautakorpi et al., 2009).

In Finland, antimicrobial consumption aligns with the European average but is higher than in other parts of Northern Europe (Goossens et al., 2007). Nevertheless, antibiotic use in children has declined across all Nordic countries, with a 36% reduction observed in Finland from 2006 to 2017 (Skajaa et al., 2022). Antibiotic overuse is the main driver of antimicrobial resistance (AMR), which has become one the leading health threats of the 21st century according to the WHO. In 2019, nearly 5 million deaths globally were associated with AMR, with 1.3 million directly attributed to drug resistance (Antimicrobial Resistance Collaborators, 2022; World Health Organization, 2023). Additionally, early childhood exposure to antibiotics is

associated with an increased risk for autoimmune diseases, such as inflammatory bowel disease, juvenile idiopathic arthritis, and asthma, as well as obesity later in life (Horton et al., 2015; Korpela et al., 2016; Kronman et al., 2012; Saari et al., 2015; Toivonen et al., 2021).

2.2 Diagnostics of Acute Infections

2.2.1 Clinical Diagnosis

Generally, children with bacterial infections present with more severe symptoms than those with viral infections (Van Den Bruel et al., 2010). The initial assessment of a febrile child (a "quick look") includes evaluating behaviour and general appearance, breathing, and body colour, with the goal of rapidly identifying critically ill children (Dieckmann et al., 2010). Further steps include taking a medical history and performing a detailed physical examination. Special attention is given to any focal findings suggesting a specific source of infection, such as tonsillitis.

Clinical warning signs for SBIs in febrile children include unconsciousness, drowsiness, inconsolability, cyanosis, increased respiratory rate or effort, prolonged capillary refill, meningeal irritation, and petechial rash (Rambaud-Althaus et al., 2015; Van Den Bruel et al., 2010; Verbakel et al., 2014). However, many of these signs are either rare (e.g., unconsciousness), non-specific (e.g., increased respiratory rate), or dependent on inter-observer agreement (e.g., capillary refill). In low-prevalence settings for serious infections, a temperature of 40°C or higher only increases the likelihood of bacterial infection from less than 1 to 5% (Van Den Bruel et al., 2010). In these settings, both parental concern and, especially, physician's intuition regarding an ill-appearing child are important alarm signals. However, common symptoms, such as cough, headache, abdominal pain, vomiting, diarrhoea, and poor feeding, have little diagnostic value in assessing the risk of bacterial infection.

No single clinical feature can rule out a bacterial infection, but certain combinations of signs can help reduce the likelihood of missing an SBI. For example, pneumonia is very unlikely if the child does not exhibit shortness of breath, and the parents are not particularly concerned (Van den Bruel et al., 2007). One study introduced a five-stage decision tree incorporating the physician's intuition, the child's age, and the presence of a temperature > 40°C, dyspnoea, or diarrhoea as a clinical decision rule with a high rule-out value for SBI (Van den Bruel et al., 2007). A well-known clinical decision rule, the Yale Observation Scale, developed in the 1980s to identify febrile children with bacterial infections, provides little value in confirming or excluding an SBI according to more recent studies (McCarthy et al., 1982; Van Den Bruel et al., 2010).

Various risk stratification criteria have been developed to detect bacterial infections in febrile infants and older children. These criteria are based on a combination of clinical signs, symptoms, and laboratory results, and include the traditional Rochester, Philadelphia, and Boston criteria developed before the pneumococcal vaccine era, as well as more recent criteria named Lab-score, Step-by-Step Approach and PECARN criteria (Baker et al., 1993; Baskin et al., 1992; Dagan et al., 1985; Galetto Lacour et al., 2008; Kuppermann et al., 2019; Mintegi et al., 2014). While the older criteria typically include information on white blood cell (WBC) count with differential, as well as urine and cerebrospinal fluid analyses, the more recent criteria involve biomarkers such as C-reactive protein (CRP) and procalcitonin (PCT) but do not require a lumbar puncture to be performed routinely. These prediction rules tend to have high sensitivity but low specificity for identifying bacterial infections. In clinical practice, rather than relying on quantifiable scoring systems, clinicians often estimate the risk for SBI based on their intuition and experience in an unstructured manner as part of their clinical decision-making.

Chest radiography is the most commonly used imaging modality to help differentiate between viral and bacterial infections of the lower respiratory tract. This is despite several guidelines advising against its routine use in children who are managed as outpatients. Chest radiography has several limitations in the diagnosis of pneumonia, including potential summation shadows (false positives), masking by other anatomical structures (false negatives), poor inter-reader agreement, and the fact that a lack of radiologic abnormalities does not exclude pneumonia (Zar et al., 2017). Lobar alveolar infiltrates strongly suggest a bacterial aetiology, whereas interstitial findings are seen in both viral and bacterial pneumonias (Virkki et al., 2002).

2.2.2 Pathogen-Based Diagnostics

2.2.2.1 Viral and Bacterial Culture

Although far from ideal, bacterial culture remains the gold standard for diagnosing bacterial infections. Blood samples are incubated to detect microbial growth, followed by Gram staining and identification techniques using mass spectrometry (MALDI-TOF) or nucleic-acid based methods (Afshari et al., 2012; Briggs et al., 2021; Carlson & Koskela, 2011). The median time to detect positive blood cultures is 15 hours, with 96% of positive blood cultures in febrile infants showing growth within 36 hours (Biondi et al., 2014). Full identification and antimicrobial susceptibility testing typically takes more than 72 hours (Afshari et al., 2012; Biondi et al., 2014; Briggs et al., 2021).

Unlike bacteria, viruses require a living host cell for replication. Viral culture involves infecting and culturing host cells, observing virus-specific cytopathic

effects under a microscope, and confirming identification with immunological methods. While it can amplify even a single viral particle, growth capacity and speed vary widely across viruses. Due to its time-consuming, labour-intensive nature and limited sensitivity, viral culture has largely been replaced by modern molecular methods (Hodinka & Kaiser, 2013; Lappalainen et al., 2011; Louten, 2017).

Culture methods can be performed on a variety of specimens, are specific, detect only live pathogens (indicating active infection), and can identify unsuspected or novel pathogens. However, they are limited to cultivable pathogens and have low sensitivity for slow-growing or intracellular pathogens (Carlson & Koskela, 2011; Lappalainen et al., 2011). Some clinically relevant viruses, such as group C rhinovirus, cannot be grown in standard cell cultures (Mahony, 2008).

The reliability of a negative blood bacterial culture result depends on sample volume, yet only half of paediatric samples meet adequate volume for reliable detection of bacteraemia (Connell et al., 2007). False negatives may also result from intermittent bacteraemia or prior antibiotic use. Inaccessible infection sites, such as the lungs, often result in negative pathogen detection even in severe cases (Jain et al., 2015).

As a result, bacteraemia is identified in only about 30% of patients with clinical sepsis (Bates et al., 1997). Similarly, nearly half of children with bacterial meningitis have negative bacterial cultures from blood and cerebrospinal fluid (Niemelä et al., 2024). Contamination is common, with 80 to 93% of positive blood bacterial cultures from febrile children at the ED resulting from contamination rather than true bacteraemia (Sard et al., 2006; Waddle & Jhaveri, 2009; Wilkinson et al., 2009).

2.2.2.2 Antigen Detection

Antigen detection methods work by directly identifying viral or bacterial structures using antibodies that recognise specific microbial antigens. Commonly used techniques include immunofluorescence, immunochromatography, and enzyme-linked immunosorbent assays, also known as enzyme immunoassays (EIAs).

In immunofluorescence, antigen-specific antibodies linked to a fluorescent dye bind to microbial antigens, emitting fluorescent light under a microscope. EIA captures soluble microbial antigens on a plate with a ”capture antibody” and detects them with an enzyme-linked ”detection antibody”, producing a visible colour change. In other types of sandwich immunoassays, the enzyme may be replaced with other signal molecules, such as fluorophores or radioactive isotopes. The immunochromatographic (lateral flow) method, used in rapid diagnostic tests like for influenza and COVID-19, utilises antibodies linked to coloured particles. When the target antigen binds to these antibodies, a visible line forms, indicating a positive result (Ginocchio, 2007; Lappalainen et al., 2011; Louten, 2017).

Antigen detection methods are widely used for the detection of respiratory and gastrointestinal viruses, hepatitis B, HIV, and group A streptococcus. In cases of complicated pneumonia, pneumococcal antigen detection in pleural fluid can be useful. However, unlike in adults, detection in urine is not applicable in children due to the high prevalence of pneumococcal nasopharyngeal colonisation (Navarro et al., 2004). Antigen detection methods are generally simple to perform, rapid, and inexpensive, but are less sensitive than tests based on nucleic acid detection. They are also unable to detect some clinically relevant viruses, such as rhinoviruses, due to the high diversity of rhinovirus types and the lack of a common antigen (Waris et al., 2017).

2.2.2.3 Nucleic Acid Detection

The accuracy and speed of viral diagnostics have greatly improved with the development of molecular amplification technologies based on PCR. Nucleic acid amplification tests (NAATs) have emerged as the gold standard, largely displacing traditional methods. NAATs have high sensitivity and specificity for identifying viruses and their subtypes, enabling the detection of some viruses that other tests cannot identify and allowing the simultaneous screening of multiple viruses (Louten, 2017; Waris et al., 2017). PCR significantly outperforms viral culture, doubling respiratory virus detection rates in young children hospitalised with acute respiratory infections (Weinberg et al., 2004).

PCR methods work by denaturating microbial DNA into single strands, binding complementary primers, and amplifying the target sequence exponentially. For RNA viruses, reverse transcription into complementary DNA is required before amplification (Louten, 2017).

Multiplex PCR assays can identify multiple pathogens, both viruses and bacteria, from a single sample. These assays are now routinely used for the detection of respiratory, gastrointestinal, and central nervous system infections, among others (Drancourt et al., 2016; Ivaska et al., 2024; Onori et al., 2014; Waris et al., 2017). Cycle threshold (Ct) value, defined as cycle count required to yield a positive signal, provides an estimate of viral load; lower Ct values indicate a higher viral load. However, the relationship between viral load and disease severity remains inconsistent (Aykac et al., 2021; Franz et al., 2010; Hasegawa et al., 2015; Wishaupt et al., 2017). Quantitative real-time PCR (qPCR) tracks PCR reactions in real time, allowing precise measurement of the initial amount of starting material. It is valuable for diagnosing opportunistic viral infections in immunocompromised patients (Louten, 2017). In qPCR, the amplified target can be detected either using a double-stranded DNA dye or, more commonly in modern diagnostic assays, with a fluorescent probe that is specific to the target sequence located between the primers.

NAATs are effective in detecting atypical respiratory bacteria, such as *Mycoplasma pneumoniae* and *Bordetella pertussis*. However, they have been less successful for the diagnosis of infections caused by invasive bacteria like pneumococcus (Nolte, 2008). Pneumococcal detection in nasopharyngeal samples often reflects asymptomatic colonisation rather than pathogenic infection. Furthermore, broad-range or universal PCR assays targeting the bacterial 16S rRNA gene have limited sensitivity for detecting bacteria in blood (Ohlin et al., 2008). Nonetheless, they can be useful for detecting bacteria in other normally sterile sites, such as cerebrospinal fluid in patients with suspected meningitis, especially in patients pretreated with antibiotics (Ivaska et al., 2024; Rampini et al., 2011). T2 magnetic resonance technology is a promising method that combines nuclear magnetic resonance and molecular detection to directly identify a predefined set of pathogens from blood samples, delivering results significantly faster than culture methods with acceptable accuracy (Lucignano et al., 2022).

PCR testing is relatively expensive, requiring specialised equipment, and skilled personnel. Despite its appreciated high sensitivity, there is a potential for false positive results due to contamination during specimen collection and processing (Louten, 2017). Additionally, PCR may yield true but clinically insignificant results. Asymptomatic virus detection is extremely common in children, requiring cautious interpretation of results (Rhedin et al., 2014). Furthermore, detecting clinically significant viruses does not rule out a concurrent bacterial infection (Levine et al., 2004; P. Shah et al., 2023).

2.2.2.4 Serology

Serological methods detect the antibody response to infection by measuring levels of immunoglobulin (Ig) M or G in serum. IgM antibodies typically rise within the first week of an acute infection and decline within 1 to 3 months. However, not all viruses trigger antibody production, and IgM levels may remain elevated long after infection. IgG antibodies increase more slowly, and a significant increase between acute and convalescent samples taken two weeks apart indicates acute infection. IgG antibodies generally persist for a lifetime, signifying immunity (Lappalainen et al., 2011). Measuring IgG avidity – the strength in which IgG antibodies bind to their target antigen – can help distinguish between recent and past infections. Low-avidity antibodies, which bind weakly, indicate a recent primary infection. In cases where both IgM and IgG antibodies are present, high-avidity antibodies suggest a reactivation rather than a primary infection (Hedman et al., 1993).

Serological testing is most valuable for diagnosing systemic infections weeks after onset in immunocompetent patients, but it is less effective for superficial infections, re-infections, or in immunocompromised hosts. Limitations in sensitivity

and specificity, along with the need for repeated sampling, limits its use in diagnosing respiratory infections. For viruses like rhinovirus with hundreds of different genotypes, clinically useful serological assays do not exist (Lappalainen et al., 2011).

2.2.2.5 Microbiome Analysis

Current diagnostic techniques are limited in scope, targeting only predefined pathogens. In contrast, microbiome analysis represents a novel approach, enabling the comprehensive characterisation of microbial communities. A widely used method is 16S rRNA sequencing, which identifies and quantifies bacteria by targeting a conserved but variable component of the bacterial ribosome, the 16S ribosomal gene. While effective for diagnosing infections caused by slow-glowing or rare bacteria, it excludes other microbes, like viruses (Church et al., 2020).

Metagenomic next generation sequencing (mNGS) offers a more comprehensive approach by analysing all genetic material (DNA or RNA) in a sample. It can simultaneously identify bacteria, viruses, fungi and parasites, while exploring the entire microbiome (Berry et al., 2020; Chiu & Miller, 2019). Its clinical utility was first demonstrated in 2014, when it diagnosed neuroleptospirosis in a 14-year-old boy with severe meningoencephalitis after conventional diagnostic methods had failed (Wilson et al., 2014). This approach allowed for a targeted antimicrobial treatment, resulting in a favourable outcome. Subsequently, in severely ill patients with central nervous system infections, mNGS of cerebrospinal fluid identified novel pathogens undetected by traditional methods in 22% of cases, and guided treatment in approximately half of them (Wilson et al., 2019).

While mNGS can theoretically detect nearly all potential pathogens in a single assay, the interpretation of results is challenging. The presence of microbial DNA or RNA does not confirm causality, especially in nonsterile samples like respiratory secretions or stool. Furthermore, human DNA dominates clinical samples, with microbial reads constituting typically < 1% of total sequences. Of these, only a subset corresponds to potential pathogens (Berry et al., 2020; Chiu & Miller, 2019). A Finnish study recently demonstrated that, following primary infection, DNA viruses persist lifelong in multiple organs and may also integrate into the human genome, even in individuals without cancer (Pyöriä et al., 2024).

The cost, time, and expertise required for mNGS currently limit its widespread adoption in clinical practice. Nevertheless, mNGS is likely to have the greatest impact in diagnosing complex cases, such as rare, polymicrobial or inaccessible infections, and for immunocompromised patients, in whom the potential spectrum of pathogens is broader (Berry et al., 2020; Chiu & Miller, 2019).

2.2.3 Biomarkers for Bacterial Infection

2.2.3.1 White Blood Cell (WBC) Count

Historically, WBC count and differential, including absolute neutrophil count (ANC), have been widely used to assess the risk of bacterial infections. In response to invading bacteria, neutrophils are mobilised from the bone marrow into circulation and directed to infection site to combat pathogens. This results in leucocytosis, primarily driven by neutrophilia. A WBC count above 15 x 10^9/L and an ANC exceeding 10 x 10^9/L are typical thresholds suggestive of bacterial infection. Additionally, the bone marrow may release immature neutrophils, known as band cells, to aid in the fight against infection. In differential analysis, this is observed as a "left shift", where the band count exceeds 1.5 x 10^9/L or the ratio of immature to total neutrophils surpasses 20% (Renko et al., 2020; Rintala & Saxén, 2011). In contrast, viral infections often decrease neutrophil count, as the immune system favours deploying lymphocytes, particularly T cells, to combat viruses.

While elevated WBC counts are more commonly associated with bacterial infections, they can also occur in viral infections or in any type of physiologic stress. Non-infectious causes of leucocytosis include trauma, autoimmune disease, haemorrhage, thromboembolism, infarction, haematologic malignancy, and steroid therapy (Woodhouse et al., 2024). In one study, only half of ED patients with moderate leucocytosis (12–25 x 10^9/L) and three quarters of those with extreme leucocytosis (> 25 x 10^9/L) were diagnosed with infectious diseases (Lawrence et al., 2007).

During infections, the extent of leucocytosis varies by pathogen. Most children with sepsis caused by pneumococcus or *E. coli* exhibit WBC count above 15 x 10^9/L, while only 19% of those with *S. aureus* septicaemia do (Peltola et al., 2006). Significant leucocytosis is also observed in half of children with adenovirus infections, but rarely in other viral infections, which more often lead to leucopenia, particularly in case of influenza. During invasive bacterial infections, WBC counts may sometimes be low due to high consumption exceeding the bone marrow's production capacity (Renko et al., 2020; Rintala & Saxén, 2011).

Nowadays, WBC count and ANC have limited utility as sole predictors of bacterial infection, since bacterial biomarkers CRP and PCT have consistently outperformed them in distinguishing between viral and bacterial infections (Andreola et al., 2007; Stol et al., 2019; Van Den Bruel et al., 2011). In a meta-analysis, WBC count demonstrated low sensitivity (58%) and moderate specificity (73%) in detecting bacterial infections (Yo et al., 2012). While it may still have value in ruling in SBI, it lacks value for ruling it out (Van Den Bruel et al., 2011). Despite their limitations, WBC count and ANC are often used alongside other biomarkers in diagnostic evaluation.

2.2.3.2 C-Reactive Protein (CRP)

CRP is a non-specific acute phase protein that serves as a valuable biomarker for bacterial infection. First identified in the 1930s, its high concentrations in the blood were observed in patients with pneumococcal pneumonia. It was found to react with the bacterial cell wall's C-polysaccharide, thus earning its name 'C-reactive' (Tillett & Francis, 1930). Primarily produced by the liver, CRP functions as a pattern recognition receptor that activates the complement system and adaptive immunity. During infections, interleukin-1 (IL-1) stimulates hepatocytes to produce IL-6, which in turn promotes CRP synthesis (Du Clos, 2000; Jaye & Waites, 1997).

Normal CRP levels in plasma are below 10 mg/L. There is no single optimal cutoff level for distinguishing between viral and bacterial infections, but values between 20 to 40 mg/L are often used to rule out bacterial infections. Higher cutoffs, such as 80 mg/L, improve the specificity of CRP measurement but at the cost of sensitivity. CRP levels begin to rise within 4 to 6 hours after bacterial infection and peak 1.5 to 3 days later. Therefore, CRP's ability to exclude a bacterial infection is influenced by the duration of the illness. A low CRP level more than 24 hours after infection onset reliably rules out a systemic bacterial infection, whereas its exclusionary value is poor within the first 12 hours (Pratt & Attia, 2007). CRP can remain low in mild localised bacterial infections (e.g., otitis media), chronic or indolent bacterial infections (e.g., tuberculosis) or during certain bacterial infections (e.g. *Bordetella pertussis* or intracellular bacteria) (Jaganath et al., 2022; Principi et al., 1986; C. Wang et al., 2021). CRP levels may also reflect disease severity and treatment response (Jaye & Waites, 1997; Renko et al., 2020; Rintala & Saxén, 2011).

Besides bacterial infections, various other inflammatory stimuli, such as viral infections, inflammatory diseases, trauma, burns and surgery, can elevate CRP levels (Jaye & Waites, 1997). Among viral infections, adenovirus is known to cause higher CRP levels compared to most other viruses. More than half of children with adenoviral infections have CRP levels exceeding 80 mg/L, whereas less than 6% of children with other common viruses show similar CRP elevations (Peltola et al., 2006). Similarly, CRP levels vary among different bacterial infections: 93% of children with pneumococcaemia have CRP levels above 80 mg/L, whereas this applies to only half of those with *S. aureus* sepsis.

CRP velocity, defined as the difference in the first two CRP measurements divided by the number of hours between tests, has recently been suggested as a tool to differentiate bacterial from viral infections. In one study, bacterial infections exhibited CRP velocities four times higher than those with viral infections (Largman-Chalamish et al., 2022).

2.2.3.3 Procalcitonin (PCT)

In healthy individuals, PCT is produced in low concentrations exclusively by the thyroid gland. During bacterial infections, IL-6 and tumour necrosis factor alpha induce its production in the liver and mononuclear cells (Assicot et al., 1993; Nijsten et al., 2000). The physiological functions of PCT remain unclear. Normal plasma levels of PCT are below 0.05 µg/L, but in invasive bacterial infections levels can rise up to 1,000-fold, with median concentrations in sepsis and meningitis reported at around 40 µg/L (Andreola et al., 2007; Gendrel et al., 1999). PCT levels begin to rise rapidly within 2 to 4 hours of infection onset, peaking between 6 to 24 hours and then gradually declining as the infection resolves (Brunkhorst et al., 1998). In sepsis, elevated PCT level serves as a marker for disease severity, septic shock, and mortality (Han et al., 2003; D. Liu et al., 2015).

Compared to CRP, PCT levels require a stronger stimulus to rise, making it less sensitive for detecting localised bacterial infections or milder cases (Al Shuaibi et al., 2013; Christensen et al., 2014; Gendrel et al., 1999). Commonly used PCT cutoffs for distinguishing bacterial from viral infections range from 0.5 to 2.0 µg/L, with the lower threshold providing excellent sensitivity and the higher one, good specificity (Andreola et al., 2007; Gendrel et al., 1999; Rossum et al., 2004; Yo et al., 2012). However, levels below 1.0 µg/L do not reliably differentiate viral from bacterial infections. Moreover, about 7% of children hospitalised for confirmed viral infections show PCT levels above 1.0 µg/L (Gendrel et al., 1999). In young febrile infants, the presence of a concurrent viral infection reduced the sensitivity of PCT in detecting an SBI when a cutoff of 0.5 µg/L was applied (Kusma et al., 2023).

Similar to CRP, factors beyond infections can elevate PCT levels, including Kawasaki disease, pancreatitis, severe diabetes ketoacidosis, shock, resuscitation, trauma, burns, and surgery (Annborn et al., 2013; Dominguez et al., 2016; D'Souza et al., 2019; Ivaska et al., 2016; Mimoz et al., 1998; Mofidi et al., 2009). However, PCT levels often decrease more rapidly than CRP in these conditions unless a bacterial superinfection occurs (Annborn et al., 2013; Mimoz et al., 1998; Reith et al., 1998; Sachse et al., 1999). Notably, unlike CRP, PCT remains unchanged in chronic inflammatory diseases, even during flare-ups (Eberhard et al., 1997; Gendrel et al., 1999). In a meta-analysis, PCT concentration of 1.1 µg/L had a sensitivity of 77% and specificity of 79% in differentiating septic patients from those with a systemic inflammatory response syndrome of non-infectious origin (Wacker et al., 2013). Physiologically, PCT levels are elevated during the first five days of life. Median values on the first postnatal day range from 1 to 11 µg/L depending on the gestational age, complicating PCT's utilisation in the diagnosis of early-onset neonatal sepsis (Fukuzumi et al., 2016).

Using a cutoff of 40 mg/L for CRP and 0.5 µg/L for PCT, discrepancies between plasma CRP and PCT levels were observed in nearly one-third of children with an

acute illness in a Finnish study (Ivaska et al., 2016). Among children with elevated PCT but low CRP levels, there was a higher prevalence of bacteraemia, fever lasting less than 12 hours, hypoxia, and haemodynamic instability. In contrast, elevated CRP with low PCT levels was more commonly associated with focal bacterial infections, inflammatory conditions, and postoperative states.

A 2017 Cochrane review of randomised controlled trials concluded that using PCT-based algorithms for managing RTIs significantly reduced antibiotic exposure, associated side effects, and mortality in adults (Schuetz et al., 2017). However, a subsequent large randomised controlled trial in the U.S. failed to show clinical benefits for PCT (Huang et al., 2018). Also, the effectiveness of PCT in guiding antibiotic treatment in children remains uncertain. For example, in a study on children hospitalised with pneumonia, applying a PCT cutoff of 0.25 µg/L to guide the initiation and duration of antibiotic treatment reduced both the number of antibiotic prescriptions and the total duration of antibiotic exposure in days (Esposito et al., 2011). Conversely, other studies have reported mixed results. In children aged 1 to 36 months presenting to the ED with fever without a source, PCT guidance had no impact on antibiotic use (Manzano et al., 2010). Similarly, while PCT guidance reduced antibiotic exposure in children presenting to the ED with lower RTIs, it did not lower the rate of antibiotic prescriptions (Baer et al., 2013).

2.2.3.4 Discriminative Performance of CRP and PCT

The ability of CRP and PCT in discriminating between viral and bacterial infections is generally similar, although PCT shows a slight advantage in certain scenarios. A meta-analysis of hospitalised children and adults reported that PCT had higher sensitivity (92 vs. 86 %) than CRP, while their specificities were similar (73 vs. 70%) for differentiating bacterial from viral infections (Simon et al., 2004). Similarly, a meta-analysis of children under 3 years of age with fever without a source found that PCT was more sensitive but less specific than CRP for detecting bacterial infections (Yo et al., 2012). Common cutoffs were 40 mg/L for CRP and 0.5 µg/L for PCT, resulting in areas under the curves (AUCs) of 0.81 and 0.84, respectively. Given its faster kinetics, PCT is more reliable than CRP for detecting SBIs in children febrile for less than 8 hours (Andreola et al., 2007).

In infants below 3 months of age, using cutoffs of 20 mg/L for CRP and 0.3 µg/L for PCT, these biomarkers achieved AUC values of 0.80 and 0.81, respectively, for detecting SBIs. For predicting invasive bacterial infections specifically, PCT outperformed CRP, with an AUC of 0.91 compared to 0.77 for CRP (Milcent et al., 2016).

In the context of paediatric pneumonia, a meta-analysis of 23 biomarkers found that none were adequate to reliably define the aetiology of pneumonia. CRP (at a concentration of 60 mg/L) and PCT (at 0.6 µg/L) performed best but with insufficient

sensitivity and specificity, around 65–70% (Gunaratnam et al., 2021). In children with diarrhoea, PCT slightly outperformed CRP in identifying bacterial causes (Korczowski & Szybist, 2004).

Table 1. Advantages and limitations of current diagnostic methods in the differentiation of viral and bacterial infections from a clinical perspective.

METHOD	ADVANTAGES	LIMITATIONS
Culture methods	Identifies new or unsuspected pathogens Allows antibiotic susceptibility testing High specificity Detects live pathogens (not just nucleic acids)	Laborious and time-consuming (may take days) Potential false-positives (e.g., contamination, colonisation) Potential false-negatives (e.g., difficult-to-culture microbes, small sample volume, small amount of viable microbe, intermittent bacteraemia, prior antibiotics) Cannot diagnose inaccessible infections
Nucleic acid amplification tests (NAATs) / polymerase chain reaction (PCR)	High analytical sensitivity and specificity Can screen for multiple (> 20) microbes simultaneously Less critical sample type and handling than culture Quantitative measurement of viral load Enables viral genotyping Rapid results (within hours) Easy to automate	Only detects targeted pathogens Not all pathogens included even in comprehensive multiplex panels Cannot distinguish between asymptomatic and pathogenic findings Potential false-positives (contamination) Relatively expensive, requires specialised equipment and skills Cannot detect past infections, or differentiate acute vs chronic Diagnostic test characteristics vary for each pathogen tested
Rapid antigen detection	Rapid results (within minutes) Can screen for multiple microbes simultaneously Inexpensive High specificity	Low sensitivity compared to PCR Limited to viruses with specific known antigens, cannot detect antigenically diverse viruses (e.g., rhinovirus) Test accuracy varies widely depending on test type
Serology	Distinguishes recent vs past infections (useful for timing of infection) IgG avidity may help distinguishing primary infection from reactivation Useful when pathogen is no longer present (e.g., late-phase illness, post-infectious complication)	Often time-consuming (results within days) May require repeated sampling Limited specificity (cross-reactivity) Unsuitable for assessing antigenically diverse viruses, superficial infections, immunocompromised hosts, or secondary re-infections
Biomarkers of bacterial infection: C-reactive protein (CRP), procalcitonin (PCT)	Rapid results (within minutes to hours) Inexpensive, easy to perform Levels rise rapidly after infection onset (CRP: 4–6 h; PCT 2–4 h) PCT: excellent sensitivity for detecting sepsis Enable evaluation of disease severity and monitoring treatment response	Sensitivity and specificity depend on cutoff values Moderate specificity for bacterial infection CRP: limited ability to rule out bacterial infection within the first 12 h of onset PCT: less sensitive for detecting localised or mild bacterial infections

2.2.4 Biomarkers for Viral Infection

2.2.4.1 Myxovirus Resistance Protein A (MxA)

2.2.4.1.1 Biology and Functions of MxA

In viral infections, the first-line antiviral response involves the production of interferons (IFNs), an essential component of innate immunity. IFNs stimulate the production of other antiviral proteins and serve as key activators of the adaptive immune response, promoting both humoral and cell-mediated immunity that develop later. In young children, the innate response is particularly important, as their adaptive immunity is still immature due to limited prior exposure to pathogens. One such IFN-induced protein is MxA, a key mediator in the antiviral defence against viruses (McNab et al., 2015).

Mx proteins were initially discovered while studying mice with genetic resistance to influenza viruses, which belong to the family of orthomyxoviruses, formerly myxoviruses. Influenza-resistant mice were found to carry a functional *MX1* gene, whereas influenza-susceptible mice had a defective gene (Horisberger et al., 1983; Staeheli et al., 1988). Among the two human myxovirus resistance proteins, MxA and MxB, MxB specifically inhibits herpesviruses and HIV, whereas only MxA exhibits broad antiviral activity (Pavlovic et al., 1990; Staeheli & Haller, 2018).

MxA is an intracellular cytoplasmic guanosine triphosphatase (GTPase). GTPases are a large family of enzymes that bind guanosine triphosphate (GTP) and hydrolyse it to guanosine diphosphate, mediating fundamental cellular processes (Gilman, 1987). Structurally, the MxA protein comprises three domains:

1. Aminoterminal (N-terminal) GTPase domain (G domain): Responsible for GTP binding and hydrolysis, critical for its antiviral properties.
2. Middle-domain (bundle signalling element, BSE): Facilitates self-assembly, connects the other two domains.
3. Carboxyterminal (C-terminal) effector domain (the stalk): Facilitates self-assembly.

MxA assembles into oligomers and forms ring-like structures, which inhibit the transcription and replication of MxA-sensitive viruses early in their life cycle by encasing viral nucleocapsid structures (Gao et al., 2011; Haller & Kochs, 2011). MxA also accelerates the apoptosis of virus-infected cells (Numajiri et al., 2006, 2011). To date, it remains unclear how MxA targets such a diverse group of DNA and RNA viruses and whether there is a common determinator in MxA sensitivity

(Haller & Kochs, 2011). Single-nucleotide polymorphisms in the *MX1* gene have been shown to influence susceptibility to viral infections in humans. For instance, a polymorphism linked to elevated MxA production has been associated with a reduced risk of severe acute respiratory syndrome coronavirus 1 (SARS-CoV-1) infection, whereas individuals carrying an inactive *MX1* variant were found to be susceptible to avian influenza A virus infections (Chen et al., 2021; Ching et al., 2010). Beyond its antiviral functions, MxA contributes to apoptosis and inhibits tumour cell motility and invasiveness (Mibayashi et al., 2002; Ronni et al., 1993).

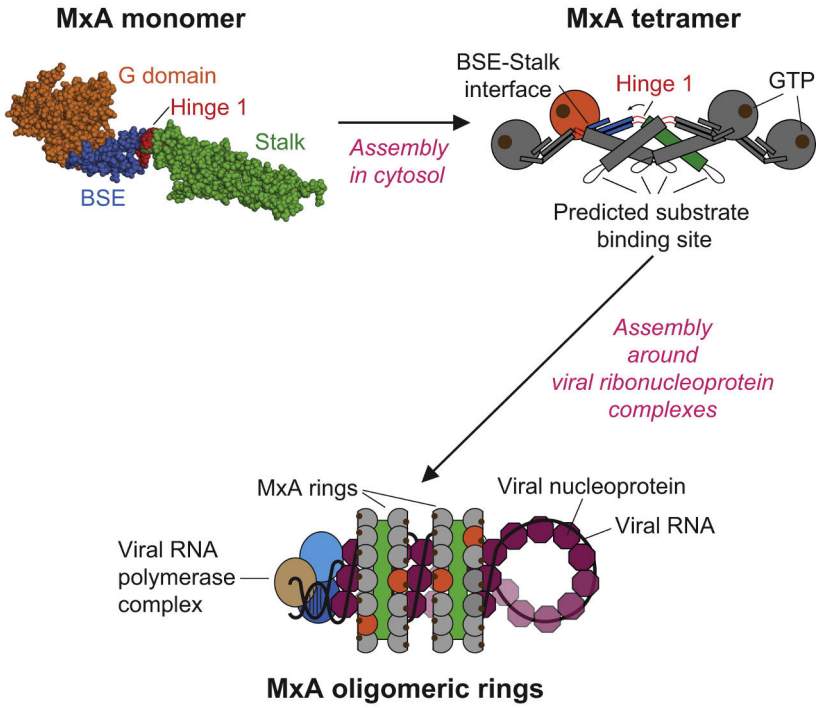

Figure 1. Structure of myxovirus resistance protein A (MxA). The MxA protein consists of three domains: aminoterminal GTPase domain (G domain), middle-domain (bundle signalling element, BSE), and carboxyterminal effector domain (the stalk). The BSE connects the G domain with the stalk via two hinge regions. MxA assembles into oligomers and forms ring-like structures that suppress the transcription and replication of MxA-sensitive viruses by encapsulating viral nucleocapsid structures. Abbreviation: GTP, guanosine triphosphate. Reprinted from (Gao et al., 2011). Used with the permission of Elsevier.

2.2.4.1.2 Regulation of MxA Expression

MxA expression is exclusively induced by type I (α, β) and III (λ) IFNs, which are characteristically produced in response to viral infections but not bacterial diseases. Being strictly dependent on IFN signalling, MxA expression cannot be directly

triggered by viruses themselves (Haller & Kochs, 2011). Furthermore, cytokines associated with bacterial infections, such as IFN II (γ), IL-1 or tumour necrosis factor alpha, do not stimulate MxA production. MxA is mainly produced in peripheral blood mononuclear cells and, to a lesser extent, locally in tissues (Ronni et al., 1993, 1997). IFN I, produced by nearly all cell types, is far more effective at stimulating the expression of MxA than IFN III, primarily produced by epithelial cells. MxA expression is regulated through the Janus-activated kinase/signal transducer and activator of transcription signalling pathway (Stark et al., 1998).

2.2.4.1.3 MxA as a Biomarker

Interferons are poor biomarkers due to their rapidly fluctuating serum levels. In contrast, MxA is a promising candidate, as it is rapidly induced within 1 to 2 hours after interferon stimulation, and with a half-life of 2.3 days, remains elevated throughout the acute phase (Ronni et al., 1993). MxA levels are low in healthy individuals, asymptomatic children who test positive for respiratory viruses, and children with bacterial infections (Engelmann et al., 2015; Nakabayashi et al., 2006; Toivonen et al., 2015).

No international standard or consensus reference range for blood MxA levels yet exists. Studies on asymptomatic individuals show that levels in children typically range from 26 to 110 µg/L, whereas the median level in adults is lower, approximately 10 µg/L (Engelmann et al., 2015; Nakabayashi et al., 2006; Rhedin et al., 2022; Toivonen et al., 2015). In addition to having higher baseline levels of MxA compared to adults, young children have been reported to exhibit stronger antiviral MxA responses compared to older ones (Rhedin et al., 2022).

Past research has identified an optimal MxA cutoff value for diagnosing viral infections in children ranging from 175 to 430 µg/L (Engelmann et al., 2015; Nakabayashi et al., 2006; Rhedin et al., 2022; Toivonen et al., 2015). This variation is influenced by factors such as the characteristics of the study population and the specific objective – whether distinguishing between viral and bacterial infections or between healthy and infected children. Mean or median MxA levels have been reported to range from 700 to 1400 µg/L in children with microbiologically confirmed symptomatic viral infections without concurrent bacterial infections (Engelmann et al., 2015; Ivaska et al., 2017; Rhedin et al., 2022; Toivonen et al., 2015). In children with pharyngitis, elevated blood MxA levels were observed in 79% of virus-positive cases, while 90% of children with streptococcal infection without viral findings had low MxA levels (Ivaska et al., 2017).

The intensity of MxA responses appears to vary slightly depending on the virus, with the highest levels observed in children with adenovirus, RSV, influenza virus, parainfluenza virus, or human metapneumovirus (median concentrations between

800–2000 µg/L). MxA levels in rhinovirus-positive children have been reported as variable, likely reflecting the frequent detection of the virus in asymptomatic children (Rhedin et al., 2022; Toivonen et al., 2015).

Studies evaluating the discriminative ability of MxA across different viral and bacterial infections, have reported AUCs ranging from 0.77 to 0.90, sensitivities from 72 to 96% and specificities between 67 and 100%, depending on the study design (Engelmann et al., 2015; Ivaska et al., 2017; Metz et al., 2023; Nakabayashi et al., 2006; Rhedin et al., 2022). Overall, MxA has shown stronger performance in studies involving children with well-defined disease entities or microbiologically confirmed infections compared to more heterogeneous populations. MxA has also been proposed as a valuable triage tool for cohorting patients with and without viral infections, thus preventing nosocomial infections in the ED during viral pandemics (Brendish et al., 2024; Mansbridge et al., 2022).

In a study of adults hospitalised with COVID-19 infection, intermediate (400–799 µg/L) or highly elevated (≥ 800 µg/L) blood MxA levels on admission were associated with a more severe course of illness (Lehtinen et al., 2022). Similarly, in infants with RSV bronchiolitis, the expression of MxA in the respiratory mucosa was higher in those infected with a severe RSV genotype compared to infants with less severe disease (Pierangeli et al., 2020). In contrast, an earlier study found no relationship between the blood MxA level and the severity of viral illness in children (Nakabayashi et al., 2006).

Despite MxA's broad antiviral activity, MxA expression has been reported to be low in small cohorts of patients with Epstein-Barr virus or cytomegalovirus infections, as both viruses employ mechanisms to suppress type I IFN signalling (T. Hu, 2025; X. Liu et al., 2020; Miller et al., 1999). In contrast, certain intracellular bacteria, such as *Mycobacterium tuberculosis*, *Chlamydia trachomatis*, and *Francicella tularensis*, are known to upregulate type I IFN production, with mycobacterial infections specifically inducing MxA production in macrophages (Boxx & Cheng, 2016; Zhou et al., 2020).

In addition to viral infections, MxA level is slightly elevated following live virus vaccinations and in active systemic autoimmune diseases involving the IFN system, such as juvenile dermatomyositis, systemic lupus erythematosus and Sjögren's syndrome (Connor et al., 2006; Huijser et al., 2019; Maria et al., 2014; Roers et al., 1994; Soponkanaporn et al., 2019; Toivonen et al., 2015). However, these rare conditions in children are not suggested to hinder the use of MxA as a marker of viral infections. Furthermore, MxA expression serves as a surrogate marker for IFN activity in patients undergoing IFN therapy for multiple sclerosis or hepatitis C (Gilli et al., 2006; Jorns et al., 2006). Importantly, MxA appears effective in diagnosing viral infections even in immunocompromised patients (Lagi et al., 2021; Metz et al., 2023; Tong-Minh et al., 2022). However, one study reported a lower sensitivity for

viral infections in immunocompromised versus immunocompetent patients (Tong-Minh et al., 2023).

In addition to measuring blood MxA levels, nasal MxA messenger RNA expression has been studied as a potential biomarker of viral RTI, offering a non-invasive alternative (Yahya et al., 2017). In a Finnish study, nasal MxA expression distinguished virus-positive children with respiratory symptoms from asymptomatic virus-negative children with good sensitivity (84%) but poor specificity (56%). Nasal MxA expression was also elevated in asymptomatic virus-positive children, limiting its utility.

2.2.5 Other Potential Biomarkers and Combinations

Numerous other protein biomarkers measuring the host response to infection have been studied. These include bacterial biomarkers such as IL-6, pro-adenomedullin, presepsin, and soluble triggering receptor expressed on myeloid cells-1, as well as the viral biomarker tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) (Woodhouse et al., 2024).

TRAIL, a membrane protein predominantly expressed on immune cells, plays a role in immune defence by inducing apoptosis in tumour cells and virus-infected cells. During viral infections, cytokines such as IFN I and II stimulate its expression (Cummins & Badley, 2009; Wei et al., 2005). TRAIL is a promising biomarker for viral infection, as its levels increase during viral infection and decrease in bacterial infections (Oved et al., 2015).

Combining multiple biomarkers appears to be more effective than relying on individual tests for differential diagnosis. A host-protein signature comprising TRAIL, interferon gamma-induced protein-10 and CRP has demonstrated superior diagnostic accuracy compared to routine biomarkers in children with RTIs or fever without a source (Oved et al., 2015; Papan et al., 2022; Srugo et al., 2017; van Houten et al., 2017). Similarly, combining MxA and CRP measurement may improve diagnostic accuracy as compared to using MxA alone (Engelmann et al., 2015).

A meta-analysis comparing two commercial biomarker combination tests for differentiating bacterial and viral RTIs reported pooled sensitivities and specificities of 87% and 82% for viral infections, and 84% and 93% for bacterial infections using MxA and CRP. For TRAIL, interferon gamma-induced protein-10 and CRP, the respective values were 90% and 92% for viral infections, and 85% and 86% for bacterial infections (Carlton et al., 2021). Notably, in a study evaluating the discriminative ability of 98 various viral and bacterial biomarker combinations in young children, 13 best-performing combinations all included MxA and TRAIL, which were two of the seven individual biomarkers assessed (Portefaix et al., 2022).

2.2.6 Artificial Intelligence (AI)

The application of artificial intelligence (AI) and machine learning (ML) in diagnosing infectious diseases has gained attention. For example, an AI system incorporating clinical data and lung lesion patterns from chest computed tomography scans accurately identified COVID-19 pneumonia and distinguished it from other types of pneumonia (K. Zhang et al., 2020). An ML model using complete blood count, CRP, age and sex differentiated viral from bacterial infections in hospitalised adults with 80% sensitivity and 85% specificity (Gunčar et al., 2024).

In a randomised clinical trial, an ML algorithm predicting sepsis in adults admitted to intensive-care units significantly reduced both mortality and length of stay by enabling blood cultures to be drawn and antibiotics to be administered nearly 3 hours earlier than in the control group (Shimabukuro et al., 2017). In the paediatric intensive-care unit setting, an ML model including four clinical and four laboratory parameters demonstrated an AUC of 0.78 in distinguishing children with sepsis from those with a non-infectious systemic inflammatory response syndrome (Lamping et al., 2018).

A study evaluating ChatGPT's responses to various infectious diseases scenarios concluded that, while it can generate diagnostic and therapeutic advice of moderate quality, it cannot replace the expertise of a clinician. The model performed best in interpreting positive blood culture results but struggled when providing guidance on bone and joint infections (Sarink et al., 2023).

Despite these promising advancements, whether AI can substantially improve the differentiation between viral and bacterial infections in febrile children at the ED using clinical signs and routine laboratory tests remains unanswered.

2.2.7 Point-of-Care (POC) Testing

2.2.7.1 Rationale and Key Features

The WHO has emphasised the need for rapid differential diagnostic tests to reduce the inappropriate use of antibiotics (O'Neill, 2016). Together with collaborators, it has proposed diagnostic performance requirements for novel tests, particularly intended for use in febrile children in low-resource settings (Dittrich et al., 2016). These guidelines recommend that an ideal test should have a sensitivity above 90% and a specificity exceeding 80% for distinguishing bacterial from non-bacterial infections. Moreover, the test should require only a small sample (50–100 µL of capillary blood), involve minimal handling, and deliver results within 10–120 minutes. Currently, no existing diagnostic method meets all these criteria.

POC tests' key features distinguish them from traditional laboratory testing. Ideally, they are performed directly at the site of care by medical personnel, require minimal training and deliver rapid results (Drancourt et al., 2016). As they utilise less specialised equipment and expertise, POC tests can also lower healthcare costs (Kokko et al., 2014). In low-income countries with limited access to conventional laboratory testing, POC tests may be the only viable diagnostic tool (Peeling & Mabey, 2010).

2.2.7.2 Microbiological POC Tests

One of the earliest and still widely used microbiological POC tests is the throat swab for detecting group A streptococcus in patients with pharyngitis (Drancourt et al., 2016). POC viral tests are primarily based on either antigen detection or, more commonly today, nucleic acid detection. Current multiplex respiratory PCR assays can detect over 20 pathogens within 1–2 hours, with some also providing Ct values (Boers et al., 2020; Leber et al., 2018; Nijhuis et al., 2017). However, their accuracy in detecting individual microbes is somewhat lower compared to respective monoplex tests (Banerjee et al., 2018; Tansarli & Chapin, 2020). Furthermore, targeted antimicrobial treatments are only available for influenza, SARS-CoV-2 and atypical bacteria detected by these assays.

The global COVID-19 pandemic created an urgent need to expand rapid diagnostic testing to improve patient management and slow the spread of the disease. Compared to NAATs, rapid antigen tests for SARS-CoV-2 offer some benefits, including faster results, lower costs, ease of use, and accessibility across diverse settings (e.g., homes, low-resource environments). Large-scale community and home-based antigen testing for SARS-CoV-2 markedly reduced COVID-19-related hospitalisations by preventing asymptomatic transmission and was estimated to achieve substantial reductions in mortality at a justifiable cost (Paltiel et al., 2021; X. Zhang et al., 2022).

Surprisingly, multiplex PCR testing for respiratory pathogens does not have a clear impact on antibiotic overuse (Abelenda-Alonso et al., 2024; Brendish et al., 2017; Busson et al., 2019; Echavarría et al., 2018; Hansen et al., 2022; Kitano et al., 2020; Kuitunen & Renko, 2023; Mattila et al., 2022; Saarela et al., 2020; Shen et al., 2021). While some studies report a reduction in antibiotic prescriptions or treatment duration, several others show no such effect. A recent multicentre study revealed that extensive molecular testing for respiratory viruses in febrile children has limited impact on diagnosis, as virus identification had poor predictive value for excluding bacterial infections (P. Shah et al., 2023). Furthermore, multiplex PCR testing does not reduce the length of stay at the ED, or the need for additional diagnostic testing, and it may increase costs (Mattila, et al., 2022). However, in patients with influenza,

POC testing allows for a more targeted antiviral use and appears to reduce antibiotic prescriptions and hospital admissions (Brendish et al., 2017; Busson et al., 2019; Green et al., 2016).

2.2.7.3 Biomarker POC Tests

In addition to pathogen-specific assays, host-response-based POC tests are also used for diagnosing acute infections. POC urine tests detect leucocytes and nitrate-reducing bacteria (Drancourt et al., 2016). POC assays for blood bacterial biomarkers WBC, CRP, and PCT are available, with CRP testing being the most extensively used.

CRP POC testing was reported to reduce antibiotic prescriptions in children with RTIs, facilitate patient flow and decrease the length of stay at the ED (Althaus et al., 2019; N. T. Do et al., 2016; N. T. T. Do et al., 2023; Hsiao et al., 2007; Kokko et al., 2014; Nijman et al., 2015; Schot et al., 2018; Smedemark et al., 2022; Verbakel et al., 2019). However, an impact on antibiotic usage is mostly seen in low- and middle-income countries with high baseline prescription rates. Two large randomised controlled trials involving children in primary care in Southeast Asia reported a significant decrease in antibiotic use in the CRP-guided group (Althaus et al., 2019; N. T. Do et al., 2016). In contrast, a French randomised study found no benefit of POC CRP testing in reducing antibiotic use in primary care outpatients (Jung et al., 2024). Likewise, in a Norwegian study, using CRP screening for all children with fever or respiratory symptoms did not reduce antibiotic prescription rate; in fact, it tended to increase it (Rebnord et al., 2016).

Two commercial assays are available for the rapid determination of blood MxA level: the Labmaster LUCIA Analyzer (Labmaster Ltd, Kaarina, Finland), which provides quantitative results, and FebriDx (Lumos Diagnostics, Sarasota, Florida), a qualitative test that combines MxA and CRP measurements. FebriDx uses a relatively low cutoff of 40 µg/L for MxA, and 20 mg/L for CRP. Most clinical data on MxA originates from studies evaluating the FebriDx. In a study involving 500 adults presenting to the ED with RTIs, FebriDx demonstrated a sensitivity of 93% and a specificity of 88% in distinguishing between bacterial and viral infections (Shapiro et al., 2022). The authors suggested that its use could reduce antibiotic overprescription from 18 to 12%. In a non-randomised feasibility study conducted in U.K. primary care in children and adults with RTI, clinicians intended to prescribe antibiotics to 86% of patients before FebriDx testing, compared to 45% after testing (Wilcox et al., 2024).

2.2.8 Gene Expression Profiling

2.2.8.1 Overview and Techniques

There is increasing interest in a novel diagnostic approach called gene expression profiling, or transcriptional analysis, which measures gene activity in cells or tissues at a given time. Unlike traditional diagnostics that rely on detecting the pathogen from the infection site, gene expression profiling assesses the host immune response, which varies depending on the pathogen. Different pathogens activate specific pattern-recognition receptors on leucocytes, resulting in unique transcriptional "biosignatures" consisting of differentially expressed genes that can be measured from a blood sample (Chaussabel et al., 2010; Gliddon et al., 2018; Ramilo & Mejías, 2009).

Gene expression profiling techniques include microarray, qPCR and RNA sequencing (RNA-seq), with RNA-seq offering the most comprehensive analysis. RNA-seq utilises next generation sequencing technologies to measure the entire set of RNA molecules produced by the host, known as the transcriptome. First, RNA is converted into a library of complementary DNA fragments, which are then amplified by PCR and sequenced. The data is analysed to quantify each RNA molecule by comparing sequence reads to a reference genome. Although RNA-seq is an excellent method for discovery science, it is labour-intensive, expensive, and involves managing large datasets, making it impractical for clinical use (Z. Wang et al., 2009). For clinical diagnosis, targeted methods like qPCR, which measure a specific set of relevant transcripts (typically fewer than 100) within a few hours, are more suitable (Holcomb et al., 2017).

2.2.8.2 Diagnostic Applications

Gene expression profiling reveals distinct transcriptional changes in patients with both systemic and localised infections (Ramilo et al., 2007; Tang et al., 2009; Zaas et al., 2009). Both viral and bacterial infections generally cause overexpression of genes related to innate immunity and suppression of adaptive immune response. However, viral infections often trigger the upregulation of IFN-inducible genes, while bacterial infections produce a more varied signature (X. Hu et al., 2013; Mejias et al., 2013; Suarez et al., 2015; Zaas et al., 2009; Zhai et al., 2015).

Gene expression signatures from peripheral blood samples have been shown to differentiate between viral and bacterial infections with high accuracy. In a proof-of-concept study by Ramilo et al. in 2007, a 35-transcript RNA signature distinguished children infected with influenza A from those with bacterial infections

with 87 to 95% accuracy (Ramilo et al., 2007). Further studies have shown similar results for other viral infections like rhinovirus and RSV (Zaas et al., 2009).

Later, two large multicentre studies evaluated the performance of transcriptional profiles in large cohorts of febrile infants and young children. Mahajan et al. identified 66 genes that best discriminated young infants with and without SBIs, and a 10-transcript biosignature that distinguished infants with bacteraemia from those without with 94% accuracy (Mahajan et al., 2016). Herberg et al. used only two transcripts to discriminate between microbiologically confirmed severe bacterial and viral infections in children with a sensitivity of 100% and a specificity of 96% (Herberg et al., 2016).

In an analysis of published host gene expression signatures aimed at distinguishing between viral and bacterial infections in both children and adults, the signature performances showed considerable variability. Median AUCs ranged from 0.69 to 0.97 for predicting viral infections and from 0.55 to 0.96 for bacterial infections (Bodkin et al., 2022). Notably, children under 11 years of age demonstrated lower diagnostic accuracy compared to adolescents and adults. Gene expression profiling can also categorise febrile children into multiple disease categories beyond just viral and bacterial infections (Habgood-Coote et al., 2023).

2.2.8.3 Challenges and Potential Benefits

Before gene expression profiling can be implemented, several challenges must be addressed. In addition to microbial pathogens, factors like age, co-morbidities, and medications may all affect transcriptional activity, complicating the analysis (Chaussabel et al., 2010). Furthermore, validating data across different time points in the disease course, various disease severities, and among patients with probable and mixed aetiologies is essential (Mahajan et al., 2013). The heterogeneity in populations and the dynamic nature of gene expression have led to inconsistent replication of the identified biomarkers in subsequent studies.

Despite the challenges, gene expression profiling has several potential advantages over traditional diagnostics. It does not require the presence of a pathogen in a sample and can help differentiate between true infections and asymptomatic shedding (Heinonen et al., 2016). It provides insights into the significance of viral-viral codetections as well as viral-bacterial coinfections (Ramilo & Mejías, 2009; Tsalik et al., 2016). Transcriptomics, together with machine learning, reliably predicted the risk of secondary bacterial infections in COVID-19 infection (Carney et al., 2024). Gene expression profiling can detect host responses during the presymptomatic phase and help assess disease severity and progression (Mejias et al., 2013; Schlapbach et al., 2024; Wallihan et al., 2018; Woods et al., 2013). Efforts to translate validated transcript signatures into feasible

POC tests were recently reported (Ko et al., 2022; Pennisi et al., 2022; Tsalik et al., 2021).

An emerging approach is exploring the transcriptome of respiratory mucosa in patients with respiratory infections. Some of the transcriptional responses observed between the respiratory epithelium and blood have been diverse (L. A. H. Do et al., 2018; Yu et al., 2019). MicroRNAs, non-coding RNAs involved in post-transcriptional regulation, are also being studied as potential diagnostic biomarkers in viral and bacterial infections (Kimura et al., 2023).

Table 2. Advantages and challenges of novel host-response diagnostic methods in the differentiation of viral and bacterial infections.

METHOD	ADVANTAGES	CHALLENGES
Biomarkers of viral infection: myxovirus resistance protein A (MxA)	Level rises rapidly (1–2 h) after infection onset Not limited to a specific set of microbes Does not require microbial components in the sample Differentiates between pathogenic and incidental virus findings POC test available	No international standard or consensus reference range for blood MxA levels Sensitivity and specificity depend on cutoff values used Cannot exclude a concurrent bacterial infection
Gene expression profiling	Not limited to a specific set of microbes Does not require microbial components in the sample Differentiates between pathogenic and incidental microbe findings Relatively simple sampling (e.g., blood) Early detection of changes in host response	Expensive and difficult to interpret Identification of a particular pathogen not possible Clinical significance of results can be difficult to determine Gene expression may be influenced by confounding factors beyond infection aetiology

3 Aims

The objectives of this thesis were to evaluate the diagnostic performance of blood MxA protein measurement, to explore the potential of host gene expression analysis in the differentiation between viral and bacterial infections in children, and to assess the accuracy of a novel rapid POC MxA test.

The specific aims were:

1. To determine the prevalence of symptomatic and asymptomatic respiratory virus infections in children hospitalised with febrile UTI and to investigate antiviral immune responses by blood MxA levels (I).
2. To evaluate blood MxA level as a biomarker for viral infection in children hospitalised with a suspected serious infection (II).
3. To assess the accuracy of a novel quantitative, rapid POC MxA test compared with the laboratory EIA in paediatric acute care and to evaluate its ability to differentiate viral from bacterial infections in febrile children presenting to the ED (III).
4. To explore whether host gene expression analysis can differentiate between viral and bacterial infections in children hospitalised for a suspected serious infection (IV).

4 Materials and Methods

Detailed descriptions of the materials and methods used are included in the original publications I–IV.

4.1 Participants and Study Design

This thesis includes four original studies with two distinct study populations. Table 3 summarises the number of children included in each study and their recruitment periods.

All studies included children aged between 4 weeks and 16 years. In Studies I, II and IV, the inclusion criteria were: 1) hospital admission, and 2) blood bacterial culture drawn by the decision of the attending physician. To ensure a balanced representation of viral and bacterial infections, we recruited a convenience sample of children meeting the following criteria: 1) hospital admission for an acute infection, 2) no prior or ongoing antibiotic treatment, and 3) venous blood samples required. Study I specifically focused on children diagnosed with febrile UTI from this population. Data on symptoms were collected by parent-filled questionnaires. For Study IV, healthy children and those with rhinoviral upper RTIs managed as outpatients were also included from an observational birth cohort study, Steps to the Healthy Development and Wellbeing of Children (STEPS).

In Study III, the inclusion criteria were: 1) fever (measured or reported ≥ 38.0°C), and 2) capillary or venous blood sample drawn by the decision of the attending physician. Children undergoing cancer treatment were excluded from all studies.

Table 3. Study populations.

	POPULATION AND SITES	RECRUITMENT
Study I	43 children hospitalised with febrile UTI (Turku University Hospital and Seinäjoki Central Hospital)	Dec 2016 – Apr 2018
Study II	265 children hospitalised with a suspected serious infection (Turku University Hospital and Seinäjoki Central Hospital)	Dec 2016 – Apr 2018
Study III	228 febrile children at the ED and paediatric ward (Turku University Hospital)	May 2020 – Sep 2022
Study IV	268 children: 211 hospitalised for a suspected serious infection (Turku University Hospital) 15 outpatients with rhinoviral infection and 42 healthy children (STEPS Study)	 Dec 2016 – Apr 2018 Nov 2010 – Mar 2012

4.2 Diagnosis of UTI (I)

In Study I, urine samples were collected via suprapubic aspiration, urine collection bag, or clean midstream specimen and analysed using flow cytometry and culture methods. A definite febrile UTI was defined as fever and a positive culture obtained through suprapubic aspiration or fever, pyuria, and a positive culture obtained through a collection bag or midstream sample. A positive culture was defined as any bacterial growth in suprapubic aspiration or $\geq 10^4$ colony-forming units/mL of a single uropathogen in other samples. Pyuria was defined as a positive leucocyte esterase test or $\geq 30 \times 10^6$ WBC/L.

4.3 Virus Detection (I–IV)

In Studies I, II and IV, nasopharyngeal swab samples were collected and suspended into phosphate-buffered saline. Nucleic acids were extracted using the NucliSENS easyMAG (bioMériuex). Multiplex reverse transcription PCR was performed using either Allplex Respiratory Panels 1–3 (Seegene) or FilmArray (Biofire Diagnostics) to detect respiratory viruses. Both methods detected adenovirus; influenza A and B viruses; parainfluenza viruses type 1, 2, 3 and 4; respiratory syncytial virus; human metapneumovirus; coronaviruses 229E, NL63, and OC43; rhinovirus; and enteroviruses. Additionally, Allplex detected human bocavirus and differentiated between entero- and rhinoviruses, while FilmArray identified coronavirus HKU1.

In Study III, nasopharyngeal samples for routine studies of respiratory viruses were collected at the discretion of the attending physician.

4.4 Other Diagnostic Measures (I–IV)

In Studies I, II and IV, blood samples for bacterial culture, WBC count, plasma CRP and PCT were collected by venous puncture and analysed by routine methods in hospital central laboratories. In Study III, CRP was measured either from a capillary blood sample using the Afinion 2 POC Analyzer (Abbott Diagnostics) or from a plasma sample by standard laboratory methods.

Additional tests and radiographic imaging were performed as deemed necessary by the attending physician.

4.5 MxA Measurements (I–III)

In Studies I and II, MxA concentrations were measured using EIA from a venous whole blood sample. In Study III, reference MxA concentrations were determined by EIA from either a capillary or venous blood sample. The samples were diluted in 1:20 hypotonic buffer and stored at -70°C until further analysis as previously described (Toivonen et al., 2015). Briefly, the EIA uses mouse anti-MxA IgG as the capture antibody and biotinylated mouse anti-MxA IgG as the detection antibody. Following immunoreaction, the signal is generated with streptavidin-peroxidase using tetramethylbenzidine as the chromogenic substrate. Recombinant human MxA, produced using a baculovirus expression system, serves as the standard. MxA concentrations are calculated from a standard curve corresponding to blood MxA concentrations of 0–1600 µg/L, with higher concentrations measured after additional sample dilution. The assay has a detection limit of 10 µg/L and requires overnight incubation in the laboratory.

The POC MxA test, used in Study III, is a sandwich immunoassay, in which the detector antibody is labelled with a Tb(III) chelate, quantified by cathodic electrochemiluminescence. It uses 7 µL of whole blood mixed with a lysis solution, applied to a test cassette, and analysed within 11 minutes. The result has a quantitative range between 50 and 1000 µg/L.

4.6 Classification of Children According to Aetiology (II–IV)

Clinical diagnoses recorded at discharge by the attending clinician formed the basis for aetiologic classification. These diagnoses were verified through a review of all available clinical, laboratory, and radiologic imaging data. In cases of diagnostic inconsistency, the final diagnosis was determined based on the expert consensus of two study physicians.

Children were initially classified into eight aetiologic groups (*detailed aetiologic classification*; Fig. 2):

1. *Definite bacterial infection*: A clinical and microbiological diagnosis of bacterial infection in the absence of viral infection.
2. *Definite viral infection*: A clinical and microbiological diagnosis of viral infection in the absence of bacterial infection.
3. *Probable bacterial infection*: Same criteria as for definite bacterial infection but without microbiological confirmation.
4. *Probable viral infection*: Same criteria as for definite viral infection but without microbiological confirmation.
5. *Viral-bacterial coinfection*: An infection with viral and bacterial aetiology, or simultaneous viral and bacterial infections at distinct foci.
6. *Bacterial infection with an asymptomatic virus finding*: A definite or probable bacterial infection and an incidental virus finding not causing symptoms.
7. *Infection of undetermined aetiology*.
8. *Non-infectious disease*.

For the statistical analyses in Study II, children with a definite or probable bacterial infection were combined into a group of *Bacterial infection*, while those with a definite or probable viral infection were categorised as *Viral infection.* This classification was also applied in Study III, where systematic respiratory virus PCR testing was not routinely performed for all children. Also, in Study III, no children were classified as having a bacterial infection with an asymptomatic virus finding.

For certain analyses in Study IV, the detailed aetiologic classification was further simplified into two groups (*dichotomous aetiologic classification*). Children with a definite or probable bacterial infection, viral-bacterial coinfection or bacterial infection with an asymptomatic virus finding were combined into a group of *Bacterial infection*. Children with a definite or probable viral infection were categorised under *Viral infection*.

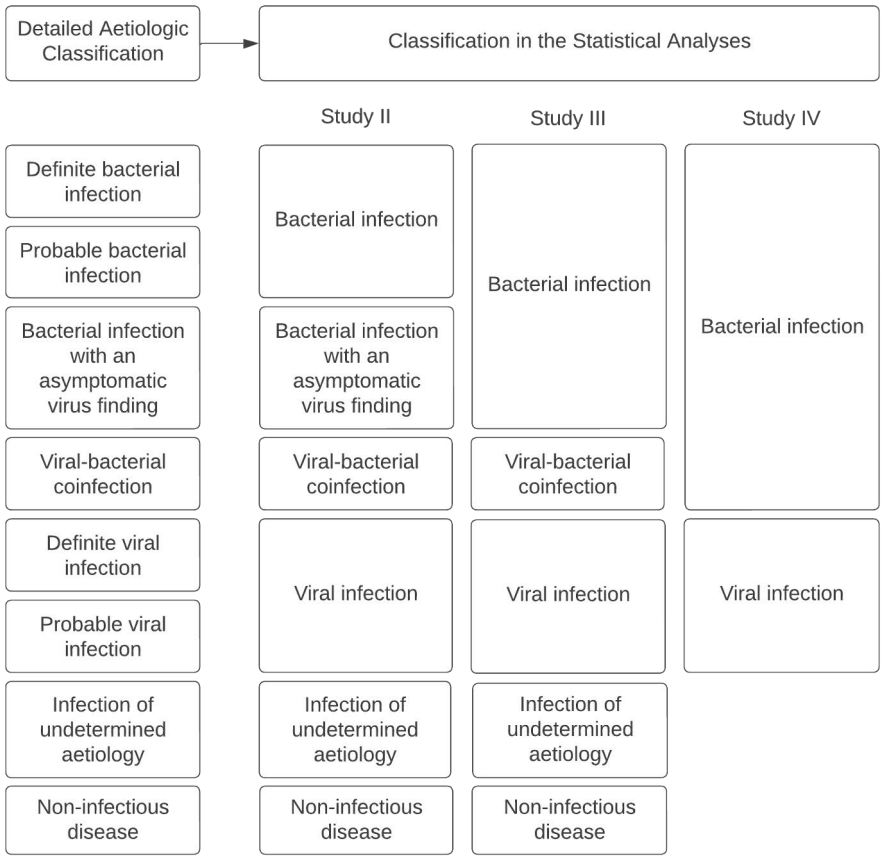

Figure 2. Classification of children according to aetiology in Studies II–IV.

4.7 Gene Expression Analyses (IV)

In Study IV, whole blood samples were collected in Tempus Blood RNA tubes (Tempus Fisher Scientific), stored at -20°C, and RNA was isolated using the Tempus Spin RNA Isolation Kit (Tempus Fisher Scientific). RNA quality was confirmed using 2100 Bioanalyzer (Agilent), with an RNA integrity number ≥ 7.5 considered suitable for sequencing. Libraries were prepared and processed with single-cell tagged reverse transcription 2 (STRT2) and STRTprep pipeline, ensuring quality check and bias correction.

Bias-corrected read counts were normalised using the spike-in normalisation method. Variable genes were then identified as those with significant fluctuations (adjusted *P* values < 0.05) beyond technical variations modelled from spike-in RNA levels. Gene expression profiles were analysed using clustering, principal component analysis, Uniform Manifold Approximation and Projection, and

heatmaps, all implemented in R. Differential expression was assessed via SAMstrt with adjusted *P* values < 0.05 indicating significance.

Logistic regression analysis was performed to predict bacterial infections based on the expression of two genes. Optimal sensitivity and specificity were estimated by Youden's method and an AUC calculated.

To validate the results with qPCR, RNA was reverse transcribed using SuperScript™ II (Invitrogen), with ERCC Spike-In Mix for normalisation. qPCR was performed using TaqMan® assays (Applied Biosystems) for TSPO (Hs00559362_m1), SECISBP2 (Hs00225345_m1), and ERCC (Ac03459873_a1). qPCR reactions were run on Bio-Rad CFX 384 thermocycler and analysed with CFX Manager 3.1. Accuracy was confirmed using log2-transformed normalised expression values.

4.8 Other Statistical Analyses

In Studies I, II and III, blood MxA levels were compared across defined aetiologic groups using the Kruskal-Wallis test, followed by Mann-Whitney U tests, with Bonferroni correction applied for multiple comparisons. Receiver operating characteristic (ROC) analysis evaluated the ability of blood MxA levels and the ratio of MxA (μg/L) to CRP (mg/L) to distinguish different aetiologic groups. Cutoff values for MxA and MxA/CRP ratio were determined by Youden index, and alternative cutoffs selected by potential clinical applicability.

In Study III, the agreement between POC and reference EIA methods for MxA levels was evaluated using the Bland-Altman method and intraclass correlation coefficients. All POC and reference test results below the POC analyser's measuring range (< 50 μg/L) were referred to as 25 μg/L and above the upper detection limit (> 1000 μg/L) as 1001 μg/L in the statistical comparison. Based on previous research, we selected a cutoff of 200 μg/L to identify an evident antiviral response, indicative of a viral infection. We then compared the proportion of children classified correctly using this cutoff with the POC analyser against results obtained from the reference method.

P values < 0.05 were considered statistically significant in all statistical comparisons. Statistical analyses were performed using SPSS (IBM).

4.9 Ethics

Study protocols were approved by the Ethics Committee of the Hospital District of Southwest Finland. The parents of all children provided their written informed consent at the enrolment.

5 Results

5.1 Respiratory Viruses and MxA in Febrile UTI (I)

Study I included 43 children hospitalised with a febrile UTI, with a median age of 4 months (interquartile range, IQR, 2–14 months). *E. coli* was the most commonly isolated pathogen from urine cultures (n = 33), followed by *Enterococcus* species (n = 4), and *Klebsiella pneumoniae* (n = 2). Respiratory viruses were identified in 40% (n = 17) of the children, with rhinovirus being the most frequent finding (n = 11). Overall, 26% (n = 11) of the children presented with respiratory symptoms, including 41% (n = 7) of virus-positive and 15% (n = 4) of virus-negative children. Thus, 16% (n = 7) of the cohort had a symptomatic, PCR-confirmed viral infection alongside UTI.

Blood MxA levels (median [IQR]) were significantly higher in virus-positive children with respiratory symptoms (778 [535–2538] µg/L) compared to either virus-negative (155 [94–301] µg/L, $P < 0.001$) or virus-positive (171 [112–331] µg/L, $P = 0.006$) children without respiratory symptoms (Fig. 3). There was no significant difference between virus-negative and virus-positive children without respiratory symptoms. All children with a symptomatic, PCR-confirmed viral infection accompanying a UTI, had elevated MxA levels, ranging from 458 to 3367 µg/L.

ROC analysis demonstrated that MxA levels effectively distinguished virus-positive children with respiratory symptoms (n = 7) from virus-negative children without respiratory symptoms (n = 22), with an AUC of 0.96 (95% confidence interval, CI, 0.89–1.0). Using a cutoff of 409 µg/L, sensitivity reached 100%, and specificity was 91%. When differentiating virus-positive children with respiratory symptoms (n = 7) from those without (n = 10), the AUC was 1.0 (95% CI, 1.0–1.0), with a cutoff of 434 µg/L yielding 100% sensitivity and specificity.

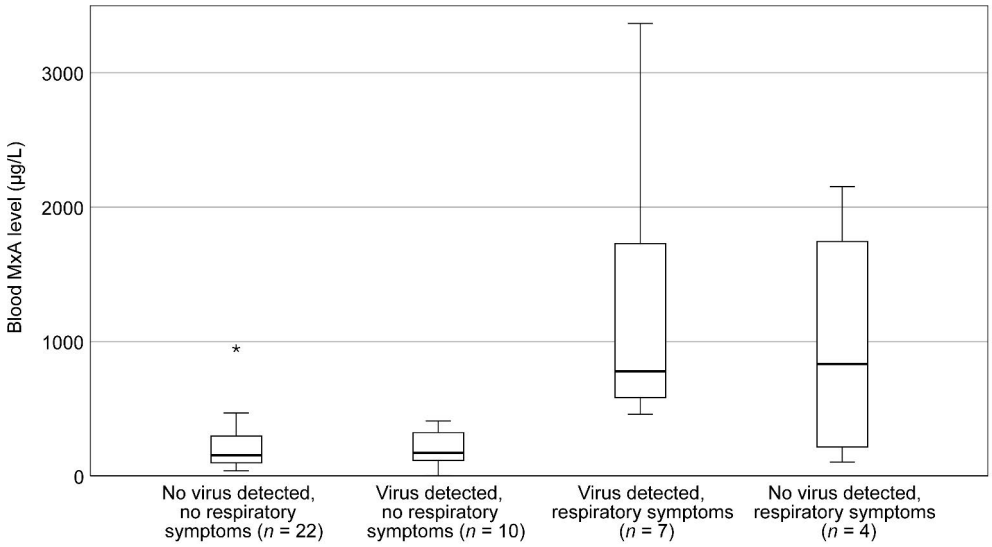

Figure 3. Blood myxovirus resistance protein A levels in 43 children with febrile urinary tract infection, according to viral detection and respiratory symptoms. In a boxplot, the horizontal line represents the median, the box indicates the interquartile range (IQR), and the whiskers represent the 95% confidence interval. An asterisk indicates an extreme value beyond 3 times the IQR. Pairwise comparisons of the group "Virus detected, respiratory symptoms" with "No virus detected, no respiratory symptoms", $P < 0.001$, and with "Virus detected, no respiratory symptoms", $P = 0.006$ (Mann-Whitney U). Modified from Study I.

5.2 MxA in Children with a Suspected Serious Infection (II)

5.2.1 Study Population

In Study II, we recruited 259 children initially suspected of having a serious bacterial infection, as indicated by the request of blood bacterial culture, along with 14 children with a suspected viral infection. After exclusions (age < 4 weeks, missing MxA samples, or samples collected > 48 hours post-admission), a total of 265 children were analysed.

Clinical characteristics, diagnoses, and detected microbes of study children are summarised in Table 4. The median age was 3.5 years (IQR, 1.3–8.1 years), with 25% having an immunosuppressive or other chronic condition. The most common diagnoses were pneumonia, pyelonephritis, and skin and/or soft tissue infections, although viral respiratory infections and generalised viral infections were also prevalent in those presenting with suspected bacterial infections. Children enrolled for a suspected viral infection mostly had upper RTIs or viral gastroenteritis. Respiratory viruses were detected in 61% ($n = 150$) of the 248 children studied, with

rhinovirus being the most common (*n* = 78; 32%), followed by RSV (*n* = 27; 11%) and human bocavirus (*n* = 20; 8%).

Table 4. Clinical characteristics, diagnoses, and detected microbes of 265 children in Study II.

CHARACTERISTIC, DIAGNOSIS, OR MICROBE		NO. (%)
Age, y, median (IQR)		3.5 (1.3–8.1)
Chronic conditions	Immunosuppressive disease or medication	7 (2.6)
	Other condition	59 (22.3)
Disease characteristics	Antibiotic treatment during hospitalisation	234 (88.3)
	Admitted to intensive-care-unit	15 (5.7)
Clinical diagnoses	Pneumonia	81 (30.6)
	Pyelonephritis	49 (18.5)
	Skin or soft tissue infection	33 (12.5)
	Viral respiratory infection[1]	22 (8.3)
	Tonsillitis	19 (7.2)
	Sepsis or toxic shock syndrome	12 (4.5)
	Central nervous system infection	8 (3.0)
	Chickenpox, herpes zoster, EBV infection, or enteroviral disease	8 (3.0)
	Gastroenteritis	7 (2.6)
	Osteomyelitis	5 (1.9)
	Infectious disease of other or undetermined aetiology	17 (6.4)
	Non-infectious disease[2]	4 (1.5)
Respiratory viruses[3]	Rhinovirus	78 (31.5)
	Respiratory syncytial virus A or B	27 (10.9)
	Human bocavirus	20 (8.1)
	Adenovirus	15 (6.0)
	Human metapneumovirus	12 (4.8)
	Influenza virus A or B	11 (4.4)
	Coronavirus V229E, NL63, OC43, or HKU1	11 (4.4)
	Parainfluenza virus 1, 2, 3, or 4	10 (4.0)
	Enterovirus	5 (2.0)
	Two or more viruses	34 (13.7)
Other viruses	Herpesviruses	10 (3.8)
	Rotavirus	3 (1.1)
Bacterial species isolated from blood or other sterile site[4]	*Streptococcus pneumoniae*	6 (2.7)
	Staphylococcus aureus	6 (2.7)
	Escherichia coli	2 (0.8)
	Haemophilus influenzae	2 (0.8)
	Streptococcus intermedius	1 (0.4)
	Salmonella paratyphi	1 (0.4)

Abbreviations: EBV, Epstein-Barr virus; IQR, interquartile range.
[1]Upper respiratory tract infection, wheezy bronchitis, laryngitis, or influenza, with or without otitis media or other localised bacterial complication.
[2]Henoch-Schonlein purpura, Kawasaki disease, or sickle cell disease.
[3]Of 248 children studied for respiratory viruses by multiplex PCR.
[4]Cerebrospinal fluid, pleural fluid, or lymph node biopsy.

When combining groups with definite or probable aetiology, 28% (n = 75) of the 265 children were found to have a bacterial infection only, and 15% (n = 39) a viral infection only. Viral-bacterial coinfections were identified in 39% (n = 103), while 10% (n = 26) had a bacterial infection with coincidental virus detection. Infections of undetermined aetiology accounted for 7%, and fewer than 2% were diagnosed with a non-infectious disease.

When combining groups with viral infections and viral-bacterial coinfections, 142 children (54%) were identified as having a symptomatic viral infection, while 105 (40%) did not, excluding cases with infections of undetermined aetiology.

5.2.2 MxA in the Differentiation Between Viral and Bacterial Infections

Blood MxA levels (median [IQR]) were significantly higher in children with a viral infection (467 [235 to 812] µg/L) than in those with a bacterial infection (119 [68 to 227] µg/L, $P < 0.001$) or a bacterial infection with a coincidental virus detection (150 [101 to 212] µg/L, $P < 0.001$) (Fig. 4). MxA levels in viral-bacterial coinfections (469 [178 to 827] µg/L) were comparable to viral infections alone ($P = 0.99$).

ROC analysis showed an AUC of 0.81 (95% CI, 0.73–0.90) for differentiating viral (n = 39) from bacterial infections (n = 75), with 74% sensitivity and 80% specificity for viral infections at a cutoff of 256 µg/L (Fig. 5). When distinguishing between children (n = 142) with symptomatic viral infections – either with or without bacterial coinfections – and children (n = 105) without symptomatic viral infections, the AUC was 0.79 (95% CI, 0.73–0.85), with 70% sensitivity and 79% specificity at the same cutoff.

The MxA (µg/L) to CRP (mg/L) ratio further improved diagnostic accuracy, achieving an AUC of 0.89 (95% CI, 0.83–0.96) for discriminating between viral and bacterial infections. At a cutoff of 19, it achieved 93% sensitivity and 77% specificity, while a cutoff of 5 provided 72% sensitivity and 88% specificity. Among 13 children with bacteraemia, all had an MxA/CRP ratio below 19, regardless of the possible presence of respiratory symptoms.

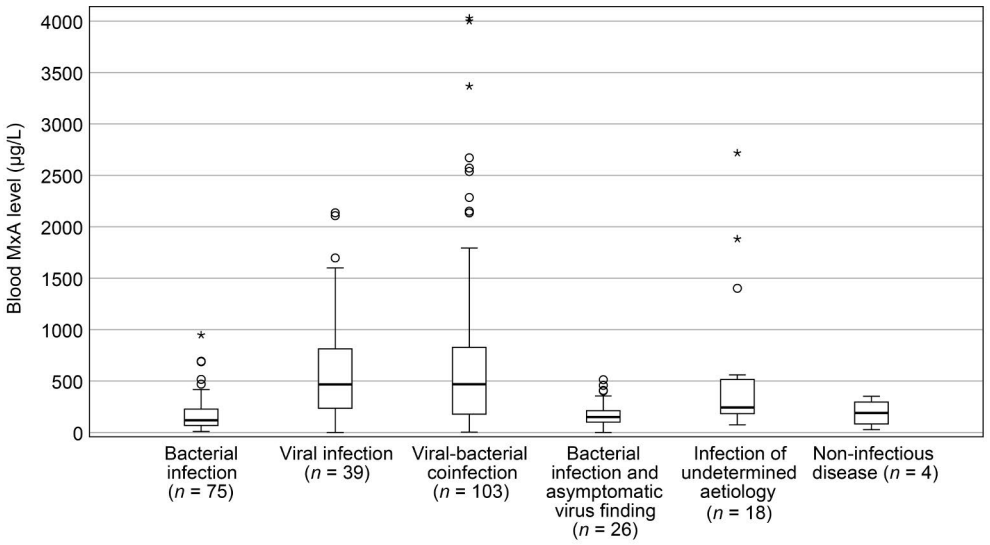

Figure 4. Blood myxovirus resistance protein A levels according to aetiology of infection. The horizontal line represents the median, the box indicates the interquartile range (IQR), and whiskers indicate the 95% confidence interval. Circles identify outliers exceeding 1.5 times the IQR, while asterisks indicate extreme values beyond 3 times the IQR. Pairwise comparisons of the groups "Viral infection" and "Viral-bacterial coinfection" against "Bacterial infection" and "Bacterial infection with coincidental virus finding" yielding $P < 0.001$ for all comparisons (Mann-Whitney U). Modified from Study II.

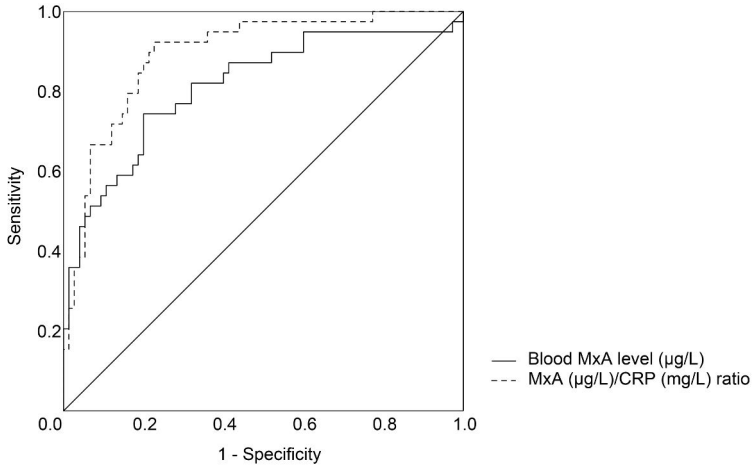

Figure 5. Discrimination between viral and bacterial infections using blood myxovirus resistance protein (MxA) level and MxA to C-reactive protein (CRP) ratio in children hospitalised with a suspected serious infection. Receiver operating characteristic (ROC) curves demonstrate the performance of MxA (µg/L) and MxA/CRP (mg/L) ratio in differentiating between viral (n = 39) and bacterial (n = 75) infections. Area under the curve was 0.81 (95% CI 0.73–0.90) for MxA and 0.89 (0.83–0.96) for MxA/CRP ratio, respectively. Modified from Study II.

5.3 POC MxA (III)

5.3.1 Study Population

In Study III, we initially recruited 248 children. After excluding cases with missing sample or result, or a delay of more than 72 hours between sampling and processing, we analysed data from 228 children. A majority (83%) of the blood samples were collected and analysed by ED personnel, while the remaining were collected at the ward and analysed by laboratory personnel.

Clinical characteristics, diagnoses, and detected microbes of study children are summarised in Table 5. The median age of children was 1.9 years (IQR, 0.8–5.4 years), and 24% (n = 55) had an immunosuppressive disease or other chronic condition. The majority of children (n = 142; 62%) were outpatients. The most common clinical diagnoses were viral respiratory infections, with or without otitis media (n = 79; 35%), undetermined viral infections (n = 29; 13%), and pneumonia (n = 21; 9%). Any viral sample was collected from 65% of children for the detection of respiratory, gastrointestinal, or herpes simplex viruses. The most frequent viral findings were SARS-CoV-2 (n = 15) and rhinovirus (n = 11).

We identified that 60% (n = 112) of the children had a viral infection, 18% (n = 34) had a bacterial infection, 10% (n = 19) had a viral-bacterial coinfection, 8% (n = 15) had an infection of undetermined aetiology, and 4% (n = 8) had a non-infectious disease.

Table 5. Clinical characteristics, diagnoses, and detected microbes in 228 children from Study III.

CHARACTERISTIC, DIAGNOSIS, OR MICROBE		NO. (%)
Age, y, median (IQR)		1.9 (0.8–5.4)
Chronic conditions	Immunosuppressive disease or medication	10 (4.4)
	Other condition	45 (19.7)
Disease characteristics	Antibiotic treatment upon discharge	85 (37.3)
	Admitted to hospital	86 (37.7)
	Admitted to intensive-care-unit	12 (5.3)
Clinical diagnoses	Viral respiratory infection[1]	79 (34.6)
	Undetermined viral infection	29 (12.7)
	Pneumonia	21 (9.2)
	Pyelonephritis	18 (7.9)
	Suspected or microbiologically verified sepsis without focus	12 (5.3)
	Tonsillitis	12 (5.3)
	Gastroenteritis	11 (4.8)
	Skin or soft tissue infection	10 (4.4)
	Osteomyelitis	5 (2.2)
	Enteroviral disease, EBV infection, or exanthema subitum	5 (2.2)
	Central nervous system infection	3 (1.3)
	Appendicitis	2 (0.9)
	Non-infectious disease or fever of unknown origin	21 (9.2)
Respiratory and other viruses	SARS-CoV-2	15
	Rhinovirus	11
	Human bocavirus	5
	Respiratory syncytial virus A or B	4
	Parainfluenza virus 1, 2, 3, or 4	4
	Herpesviruses[2]	4
	Other[3]	11
Bacterial species isolated from blood, other sterile site[4], or urine	*Escherichia coli*	15
	Streptococcus spp (*S. pneumoniae, agalactiae, or anginosus*)	4
	Pseudomonas aeruginosa	3
	Staphylococcus aureus	3
	Other[5]	6

Abbreviations: EBV, Epstein-Barr virus; IQR, interquartile range; SARS-CoV2, severe acute respiratory syndrome coronavirus 2.
[1]Upper respiratory tract infection, bronchiolitis, wheezy bronchitis, laryngitis, SARS-CoV-2 infection, or influenza with or without otitis media or other localised bacterial complication.
[2]Herpes simplex virus or EBV (*n* = 2 each).
[3]Human metapneumovirus (*n* = 3); adenovirus (*n* = 3); influenza virus A (*n* = 2); coronavirus 229E (*n* = 1); enterovirus (n = 1), astrovirus (n = 1).
[4]Cerebrospinal fluid, pleural fluid, or abscess fluid.
[5]*Enterococcus faecalis*, *Aerococcus urinae*, *Enterobacter cloacae* complex, *Klebsiella pneumoniae* complex, *Veillonella parvula*, or *Haemophilus parainfluenzae*

5.3.2 Accuracy and Feasibility of POC MxA Measurement

The mean difference between the POC and reference MxA levels was -76 µg/L, with 95% limits of agreement ranging from -409 to 257 µg/L (Fig. 6a). The intraclass correlation between the two methods was 0.878 (95% CI, 0.799–0.921) (Fig. 6b). At a cutoff of 200 µg/L, indicating an antiviral response, 87% of the POC MxA results were consistent with the reference method. Of the 111 POC MxA values above the cutoff, 98% matched the reference assay.

In a sensitivity analysis, when POC results (n = 35) exceeding the POC analyser's measuring range (1000 µg/L) were excluded, the mean difference was -96 µg/L (95% limits of agreement from -429 to 237 µg/L). In children with POC values over 1000 µg/L, the reference method showed results ranging from 481 to 4684 µg/L (median, 2264 µg/L). In another sensitivity analysis limited to children recruited at the ED (n = 188), the agreement between POC and reference tests remained similar.

POC testing required approximately 2 minutes for sample collection and preparation (dilution and transfer to a test cassette), followed by 11 minutes for processing with the LUCIA Analyzer. Thus, results were available within 15 minutes of starting the test. Technical errors were rare. ED personnel generally gave positive feedback on the POC test. Negative feedback included the time required for sample preparation and reading of a near-field communication card before each analysis.

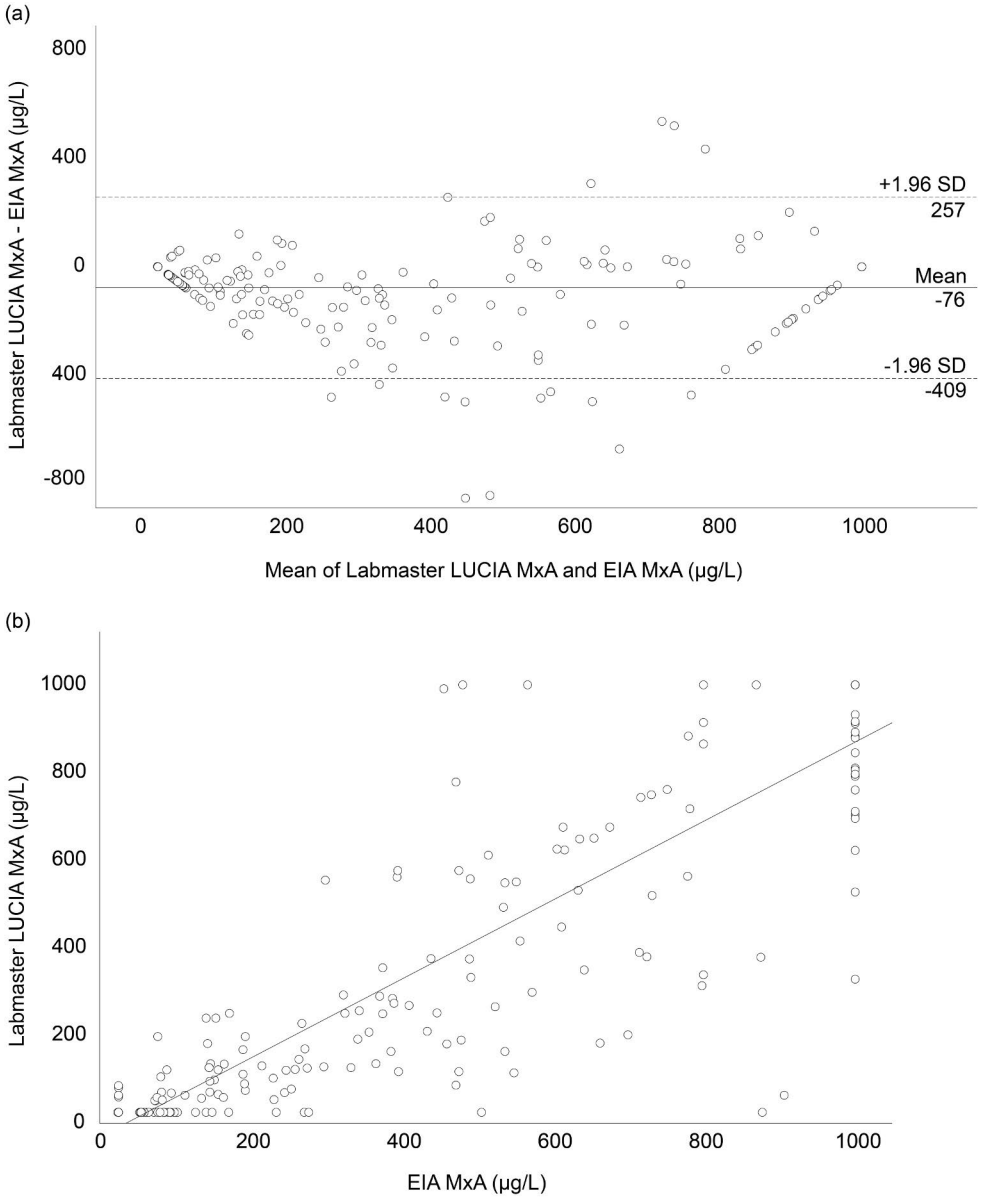

Figure 6. Bland-Altman and linear correlation plots comparing blood myxovirus resistance protein A (MxA) levels measured by point-of-care (POC) and reference methods in 228 febrile children. (a) Bland-Altman plot illustrates the agreement between the POC Labmaster LUCIA MxA measurement and reference enzyme immunoassay (EIA) analysis. The solid line represents the mean difference (-76 µg/L) between the methods, with dashed lines showing the 95% limits of agreement (from -409 to 257 µg/L). (b) Scatterplot shows the linear correlation between the POC Labmaster LUCIA MxA measurement and reference EIA results. Abbreviations: EIA, enzyme immunoassay; MxA, myxovirus resistance protein A; SD, standard deviation. Modified from Study III.

5.3.3 POC MxA in the Differentiation Between Viral and Bacterial Infections at the ED

Among 188 children recruited at the ED, POC MxA levels (median [IQR]) were significantly higher in those with a viral infection (571 [240–955] µg/L; n = 112) compared to those with bacterial infections (25 [25–54] µg/L; n = 34; P < 0.001) (Fig. 7). In children with a viral-bacterial coinfection (n = 19), MxA levels were similar (555 [103–889] µg/L; P = 1.00) to children with a viral infection only.

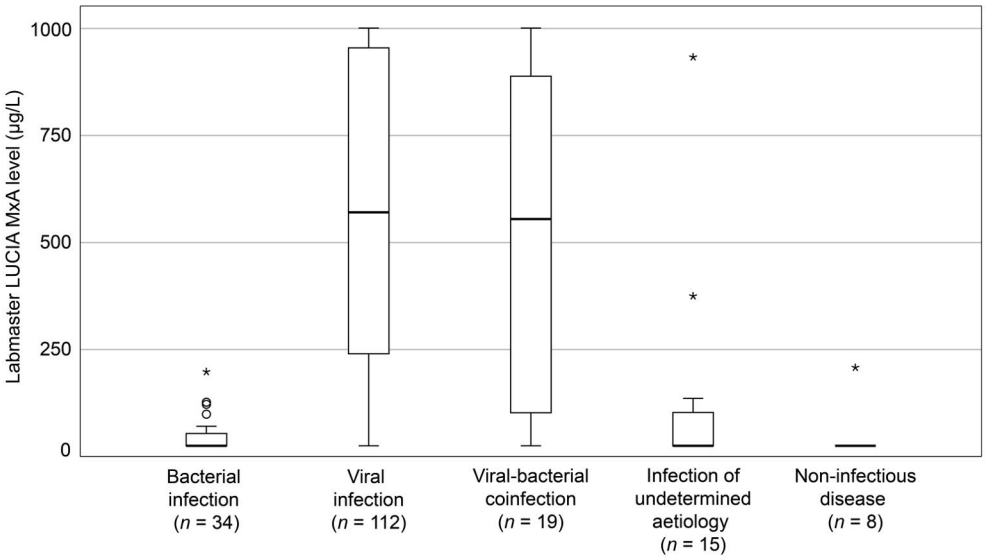

Figure 7. Point-of-care Labmaster LUCIA blood myxovirus resistance protein A levels in 188 febrile children at the emergency department, grouped by aetiology. For each group, the horizontal line indicates the median, the box represents the interquartile range (IQR), and the whiskers mark the 95% confidence interval. Circles identify outliers exceeding 1.5 times the IQR, while asterisks indicate extreme values beyond 3 times the IQR. Pairwise comparisons of the groups "Viral infection" and "Viral-bacterial coinfection" against "Bacterial infection" yielded P < 0.001 for both comparisons (Mann-Whitney U test). Modified from Study III.

In a ROC analysis, POC MxA levels effectively differentiated viral from bacterial infections, with an AUC of 0.96 (95% CI, 0.94–0.99) (Fig. 8). The optimal sensitivity (92%) and specificity (91%) were achieved at a cutoff of 101 µg/L, while a cutoff of 200 µg/L gave a sensitivity of 79% and specificity of 100%. When combined with CRP measurement, the MxA/CRP ratio achieved an AUC of 0.97 (95% CI, 0.95–0.99), with the best sensitivity (83%) and specificity (100%) at a cutoff of 11.

Among the ED-recruited children, 21% (n = 40) had a microbiologically confirmed aetiology of infection. POC MxA levels were higher in those with a

confirmed viral infection (785 [558–1001] µg/L; *n* = 18) than in those with a confirmed bacterial infection (25 [25–25] µg/L; *n* = 17; *P* < 0.001). In children with a confirmed viral-bacterial coinfection, MxA levels were intermediate (251 [59–562] µg/L; *n* = 5; *P* = 0.074 and *P* = 0.14 when compared to confirmed bacterial and viral infections, respectively). ROC analysis showed AUCs of 0.96 (95% CI, 0.89–1.00) for POC MxA and 0.99 (95% CI, 0.97–1.00) for the MxA/CRP ratio, with optimal cutoffs of 232 µg/L and 15, respectively.

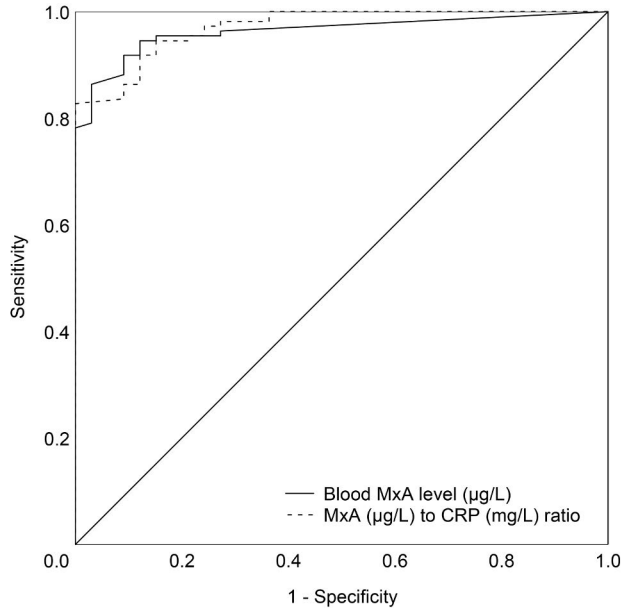

Figure 8. Discrimination between viral and bacterial infections using point-of-care blood myxovirus resistance protein A (MxA) level and MxA to C-reactive protein (CRP) ratio in febrile children at the emergency department. Receiver operating characteristic curves demonstrate the performance of MxA (µg/L) and MxA (µg/L)/CRP (mg/L) ratio for distinguishing between viral (*n* = 112) and bacterial (*n* = 34 for MxA and n = 33 for MxA/CRP) infections. Area under the curve was 0.96 (95% confidence interval [CI], 0.94–0.99) for MxA, and 0.97 (95% CI, 0.95–0.99) for MxA/CRP ratio, respectively. Modified from Study III.

5.4 Effects of Age and Live Vaccinations on MxA Levels (I–III)

Studies II and III demonstrated a significant influence of age on MxA levels. In Study II, among hospitalised children without a symptomatic viral infection, those under 2 years of age (*n* = 39) had higher median (IQR) MxA level (160 [112–306] µg/L) compared to children aged 2 years or older (109 [52–186] µg/L; *n* = 66; *P* = 0.003). Similarly, in children with a symptomatic viral infection (with or without a

bacterial coinfection), MxA level was higher in children under 2 years (614 [209–1090] µg/L; $n = 50$) compared to older children (403 [170–716] µg/L; $n = 92$; $P = 0.045$). In Study III, in ED-recruited children with a viral infection (with or without a bacterial coinfection), POC MxA level was higher (704 [299–1001] µg/L) in children under 2 years of age ($n = 81$) compared to older children (292 [164–776] µg/L; $n = 50$; $P < 0.001$). In Study I, among children with febrile UTI without concomitant respiratory symptoms, MxA level was slightly higher in those under 12 months of age (186 [126–322] µg/L; $n = 25$) than in those aged 12 months or older (129 [66–191] µg/L; $n = 7$), although this difference was not statistically significant ($P = 0.074$).

In Study II, we examined the impact of recent live vaccinations on blood MxA levels in young children under 2 years old who did not have a symptomatic viral infection. No significant difference was observed in MxA levels (median [IQR]) between children who had recently received a live-virus vaccine (186 [150–321] µg/L; $n = 14$) and those without prior vaccination (143 [99–282] µg/L; $n = 25$; $P = 0.26$).

5.5 Gene Expression in Viral and Bacterial Infections (IV)

5.5.1 Study Population

In Study IV, RNA samples were analysed from 268 children, including 211 hospitalised for a suspected serious infection, 15 outpatients with a confirmed rhinoviral respiratory tract infection, and 42 healthy children from the STEPS Study. The median age was 3.4 years (IQR, 1.6–7.9 years) for children with acute infections and 2.1 years (IQR, 1.2–2.1 years) for healthy children. Among the 226 children with acute infections, 22% ($n = 49$) had immunosuppressive or other chronic conditions. Respiratory viruses were detected in 61% ($n = 122$) of hospitalised children of 200 studied, and all outpatients, with rhinovirus, RSV, and human bocavirus being the most common.

Among the children with acute infections, 15% ($n = 34$) were diagnosed with a definite bacterial infection, 13% ($n = 29$) with a probable bacterial infection, 34% ($n = 77$) with a viral-bacterial coinfection, and 10% ($n = 23$) with a bacterial infection and an asymptomatic virus finding. Definite viral infections accounted for 18% ($n = 41$), with probable viral infections at 3% ($n = 7$). Additionally, 5% had an infection of undetermined aetiology, and 1% a non-infectious disease.

5.5.2 Clustering Based on Blood Transcriptome

We first compared the gene expression profiles of all 268 study children based on the detailed aetiologic classification, detecting 15,789 protein-coding genes, 6,308 of which were significantly variable. Using these, we identified five clusters, but the three main aetiologic groups (definitive bacterial, definite viral, and control) were not the primary determinants of clusters (Fig. 9). Principal component analysis and Uniform Manifold Approximation and Projection revealed distinct patterns between healthy children and those with bacterial infections. However, children with viral infections exhibited heterogeneous expression profiles that were not clearly distinguishable from others.

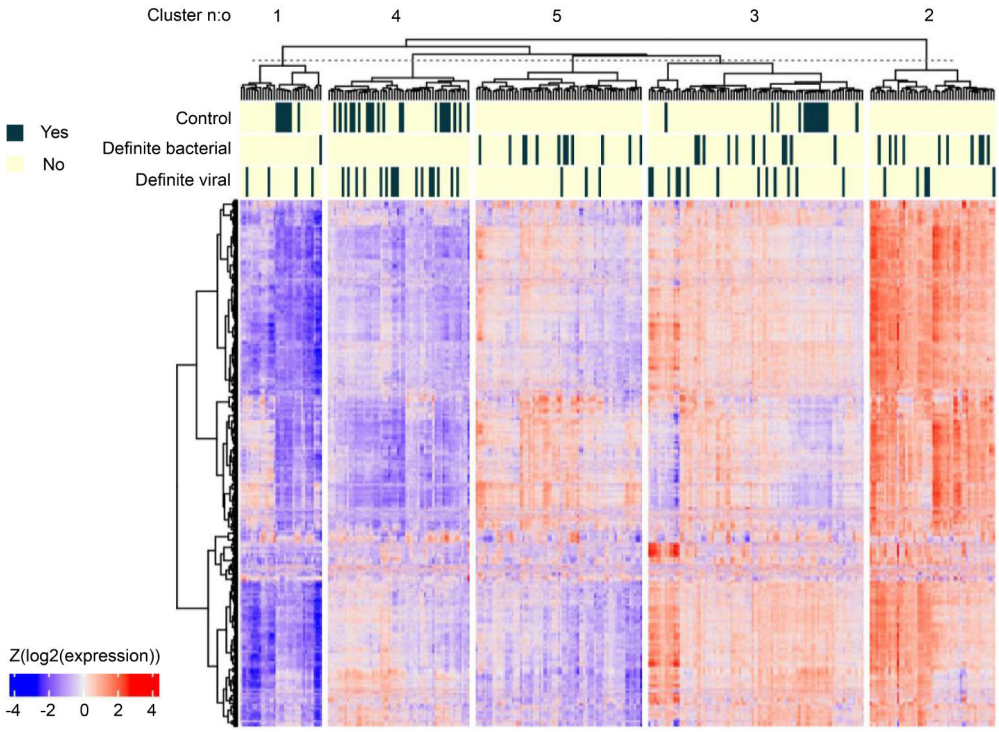

Figure 9. Overview of peripheral blood gene expression profiles in 42 healthy control children, 34 children with a definite bacterial infection, and 41 children with a definite viral infection. The heatmap, created using k-means clustering, displays patients as columns and RNA transcripts as rows. Colours indicate gene expression intensity; red signifies upregulated RNA transcripts, while blue represents downregulated transcripts. Modified from Study IV.

5.5.3 Characteristics of Discovery and Validation Groups

To reduce the heterogeneity in the study population, we stratified the cohort into a discovery group (n = 101) of children with respiratory tract infections and a validation group (n = 109) with non-respiratory infections, classified according to the dichotomous (bacterial vs. viral) aetiology. In the discovery group (median age, 2.9 years) 71% of children had bacterial infections, while 29% had viral infections. Clinical diagnoses in this group included pneumonia (n = 66) and upper or lower respiratory tract infections without pneumonia (n = 35). The validation group (median age, 5.0 years) was predominantly (84%) bacterial, with a smaller proportion diagnosed with viral infections. The two most common clinical diagnoses in this group were pyelonephritis (n = 36) and skin or soft tissue infections (n = 28).

5.5.4 Differentiation Between Bacterial and Viral Infections by 2-Transcript Signatures

In sequencing the discovery group, 6,147 genes were identified, with 2,974 showing differential expression between bacterial and viral respiratory infections. The best 2-transcript predictor for bacterial infection was the combination of *GAPDH* and *SHQ1* genes, with an AUC of 0.96 (95% CI, 0.93–0.99), sensitivity of 89%, and specificity of 100% (Table 6, Fig. 10). However, this combination performed less well in the validation group, and yielded an AUC of 0.79 (95% CI, 0.73–0.86) when applied to both the discovery and validation groups. The most effective signature in the validation group alone was *C12orf75* and *TUSC2* (AUC 0.88), but it still showed reduced discriminative power in the combined groups (AUC 0.81).

A more robust 2-transcript signature, consisting of *TSPO* and *SECISBP2* genes, performed strongly across the discovery, validation and combined groups, with AUCs of 0.93 (95% CI, 0.88–0.98), 0.81 (95% CI, 0.71–0.91) and 0.87 (95% CI, 0.82–0.92), respectively (Table 6, Fig. 10). In the discovery group, the signature achieved a sensitivity of 88% and specificity of 90%, while in the validation group, sensitivity was 71% and specificity 89%. In the combined groups, sensitivity reached 77% with 87% specificity. When prioritising a higher sensitivity of 85% to improve the rule-out capability for bacterial infections, specificity in the combined groups was 70%. *TSPO* expression was elevated in bacterial infections and reduced in viral infections, whereas *SECISBP2* exhibited the opposite pattern. This signature outperformed CRP and PCT in distinguishing between bacterial and viral infections, despite their partial use in the aetiologic classification.

Lastly, the signature proposed by Herberg et al. which includes the genes *IFI44L* and *FAM89A* and demonstrated excellent performance in their cohort of children with microbiologically confirmed severe bacterial and viral infections, showed poor discriminative ability in our study population (Herberg et al., 2016).

We internally validated the top-performing 2-transcript bacterial infection predictor, *TSPO* and *SECISBP2*, by qPCR in 90 samples (61 from the discovery group and 29 from the validation group). The gene pair distinguished bacterial from viral infections with an AUC of 0.82 (95% CI, 0.72–0.91) across the combined dataset.

Table 6. Performances of selected 2-transcript signatures and bacterial biomarkers for predicting bacterial infection.

		AUC (95% CI)	SENSITIVITY (%)	SPECIFICITY (%)
Discovery group: top 3 signatures	*GAPDH* & *SHQ1*	0.96 (0.93–0.99)	89	100
	ZFAND1 & *SRA1*	0.95 (0.91–0.99)	92	93
	SHQ1 & *SAR1B*	0.95 (0.91–0.99)	89	93
Discovery group: other selected signatures and biomarkers	*TSPO* & *SECISBP2*[1]	0.93 (0.88–0.98)	88	90
	C12orf75 & *TUSC2*[2]	0.82 (0.74–0.91)	76	86
	IFI44L & *FAM89*[3]	0.64 (0.51–0.76)	83	52
	CRP	0.88 (0.80–0.96)	83	87
	PCT	0.86 (0.77–0.94)	71	100
Validation group: top 3 signatures	*C12orf75* & *TUSC2*	0.88 (0.80–0.96)	80	89
	C12orf75 & *GPAA1*	0.88 (0.81–0.94)	74	100
	CDCA7 & *DHCR7*	0.88 (0.80–0.95)	74	94
Validation group: other selected signatures and biomarkers	*TSPO* & *SECISBP2*[1]	0.81 (0.71–0.91)	71	89
	GAPDH & *SHQ1*[4]	0.46 (0.33–0.60)	37	83
	IFI44L & *FAM89A*[3]	0.71 (0.56–0.86)	85	61
	CRP	0.80 (0.70–0.89)	65	94
	PCT	0.63 (0.51–0.75)	45	94
Combined groups: top 3 signatures	*TSPO* & *SECISBP2*	0.87 (0.82–0.93)	77	87
	CD44 & *SECISBP2*	0.87 (0.81–0.93)	83	81
	SNX3 & *SECISBP2*	0.86 (0.81–0.91)	74	87
Combined groups: other selected signatures and biomarkers	*GAPDH* & *SHQ1*[4]	0.79 (0.73–0.86)	60	92
	C12orf75 & *TUSC2*[2]	0.81 (0.75–0.87)	68	87
	IFI44L & *FAM89A*[3]	0.61 (0.52–0.71)	71	55
	CRP	0.83 (0.76–0.89)	73	91
	PCT	0.73 (0.66–0.81)	55	94

Abbreviations: AUC, area under the curve; CI, confidence interval; CRP, C-reactive protein; PCT, procalcitonin.
[1]Best 2-transcript predictor in the combined discovery and validation groups.
[2]Best 2-transcript predictor in the validation group.
[3]Based on (Herberg et al., 2016).
[4]Best 2-transcript predictor in the discovery group.

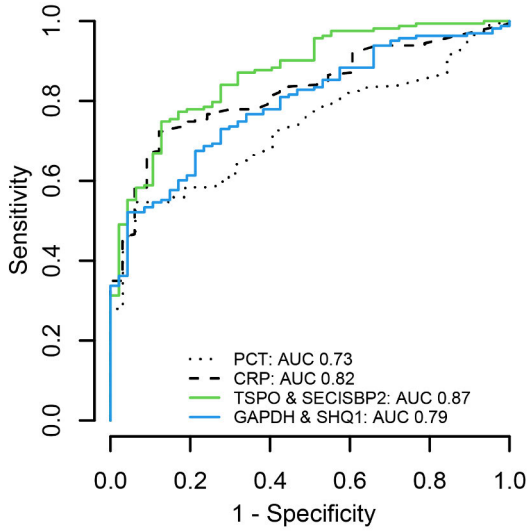

Figure 10. Receiver operating characteristic (ROC) curves for predictors of bacterial infection. The ROC curves illustrate the performance of the 2-transcript signatures (*TSPO* and *SECISBP2*, *GAPDH* and *SHQ1*), as well as C-reactive protein (CRP) and procalcitonin (PCT) for distinguishing bacterial infections in the combined discovery and validation groups. Area under the curve values (95% confidence interval, CI) were 0.87 (95% CI, 0.82–0.92) for *TSPO* and *SECISBP*, 0.79 (95% CI, 0.73–0.86) for *GAPDH* and *SHQ1*, 0.82 (95% CI, 0.76–0.89) for CRP and 0.73 (95% CI, 0.66–0.81) for PCT, respectively. Modified from Study IV.

6 Discussion

6.1 Rationale for Study Design

We adopted a pragmatic approach in our studies by including children with definite, probable and mixed aetiologies in our analyses. This design addresses a gap in previous research on MxA or gene expression profiling for distinguishing viral from bacterial infections, where complex yet clinically relevant populations were often excluded (Engelmann et al., 2015; Habgood-Coote et al., 2023; X. Hu et al., 2013; Ko et al., 2023; Nakabayashi et al., 2006; Ramilo et al., 2007; Rao et al., 2022; Ravichandran et al., 2021; Self et al., 2017; Shapiro et al., 2018; Sweeney et al., 2016). While our approach introduced heterogeneity in patient groups and some uncertainty in the aetiologic classification, potentially reducing the performance of the biomarkers studied, it also enhances the robustness and generalisability of our findings.

This design reflects real-world clinical practice, where the challenge does not lie in distinguishing asymptomatic children from febrile ones, nor differentiating between microbiologically confirmed bacterial and viral infections, but in managing the less straightforward cases. There is a need for a tool that can accurately and promptly determine the cause of febrile illness, particularly in high-pressure ED settings.

6.2 Viral-Bacterial Coinfections

In Study II, respiratory viruses were frequently detected by multiplex PCR in children hospitalised with suspected serious infections. Over 60% of these children tested positive for at least one respiratory virus, with nearly 40% classified as having a symptomatic viral-bacterial coinfection. In Study I, among children with febrile UTI, 40% were positive for respiratory viruses, although only half of virus-positive exhibited respiratory symptoms. Study III found that 10% of febrile children at the ED had a symptomatic viral-bacterial coinfection. Overall, our findings emphasise the high prevalence of viral-bacterial coinfections, particularly among hospitalised children.

Respiratory viral infections are generally associated with a lower risk of UTI and SBI in febrile infants compared to virus-negative children, though this relationship varies by virus type (Byington et al., 2004; Mahajan et al., 2018). For instance, the presence of RSV or influenza virus has been linked to a lower likelihood of UTI (Krief et al., 2009; Levine et al., 2004), whereas rhinovirus-positive infants show a risk comparable to virus-negative infants (Blaschke et al., 2018). This discrepancy is likely due to the high detection rate of rhinovirus in asymptomatic children.

Demonstrating antiviral immune response by MxA measurement could help identify virus-positive infants with a truly pathogenic viral infection, who may have a lower risk of SBI, as opposed to those with an incidental asymptomatic viral detection, where the cause of fever requires further investigation. In our studies, MxA levels were low in children with coincidental virus detection during bacterial infections. Nonetheless, given that the risk of bacterial infection is non-negligible even in infants with symptomatic viral infections, MxA results – like other biomarkers – must be interpreted alongside the patient's clinical presentation and individual risk factors. Given the declining incidence of SBIs, balancing the risks of missed SBIs against unnecessary antibiotic use is increasingly important.

The prevalence of viral-bacterial coinfections presents challenges for the development of novel diagnostic biomarkers. Recognising this, Study IV grouped children with bacterial infections and viral-bacterial coinfections into a single category to define a promising 2-transcript biosignature for guiding antibiotic treatment.

6.3 MxA (I–III)

6.3.1 Performance and Optimal Cutoff

Our studies confirm the robust potential of blood MxA protein as a biomarker for viral infection in children in various clinical scenarios. In Study I, involving children with febrile UTI, elevated blood MxA levels correlated strongly with the presence of simultaneous symptomatic respiratory viral infections. This yielded high diagnostic accuracy (AUC 0.96) in differentiating virus-positive children with respiratory symptoms from virus-negative children without respiratory symptoms, reflecting MxA's ability to detect a viral infection in children with a simultaneous SBI. In Study II, among children hospitalised for suspected serious infections, blood MxA levels distinguished viral from bacterial infections with moderate accuracy (AUC 0.81). In Study III, focused on febrile children at the ED, POC testing of blood MxA level showed excellent performance (AUC 0.96), with sensitivity and specificity exceeding 90%.

The superior performance of MxA in ED-children compared to hospitalised ones is likely attributed to differences in patient populations. ED-children were primarily managed as outpatients for common childhood infections, where clinical judgement and determining infection aetiology tends to be more straightforward. In contrast, hospitalised children presented with severe illnesses, complex clinical scenarios, and frequent coinfections. In such cases, definitive aetiological classification can remain challenging, even after comprehensive diagnostic workups.

Previous studies have demonstrated excellent accuracy of MxA in distinguishing viral from bacterial infections (Engelmann et al., 2015; Metz et al., 2023; Nakabayashi et al., 2006). A Swedish study evaluating children with lower respiratory tract infections similarly reported strong diagnostic performance (AUC 0.90) (Rhedin et al., 2022). However, consistent with our findings, MxA was ineffective at distinguishing viral infections from viral-bacterial coinfections. Although a large French study assessing the discriminative performance of 98 combinations of viral and bacterial biomarkers identified MxA as a key component in the top 13 combinations, the study's grouping of bacterial infections and viral-bacterial coinfections into a single category led to a high sensitivity but low specificity for differentiating viral from bacterial infections (Portefaix et al., 2022). While highly effective at identifying symptomatic viral infections, the use of MxA as a sole biomarker is limited in settings with high rates of viral-bacterial coinfections. Combining MxA with CRP improves diagnostic accuracy in these contexts.

Optimal MxA cutoffs varied across our studies, ranging from 101 to 409 µg/L, reflecting differences in study designs and populations. The highest cutoff (409 µg/L) was observed in Study I, which primarily included infants, with a median age of 4 months. Studies II and III, along with previous research indicate that younger children (under 1–2 years of age) have both higher baseline MxA values and a more robust antiviral MxA response compared to older children (Rhedin et al., 2022). This suggests that implementing age-stratified cutoff levels for interpreting MxA results might be reasonable. The difference between optimal cutoffs for hospitalised children (256 µg/L) versus ED-children (101 µg/L) likely reflects the above-mentioned differing clinical contexts and levels of diagnostic uncertainty in these populations.

In earlier studies the optimal MxA cutoff for children has ranged from 175 to 430 µg/L, influenced by the population's characteristics. For instance, Engelmann et al. and Toivonen et al. established cutoffs of 200 and 175 µg/L, respectively, by comparing children with confirmed viral infections to healthy asymptomatic children (Engelmann et al., 2015; Toivonen et al., 2015).

Determining an optimal cutoff for MxA in children with lower respiratory tract infections or pneumonia presents particular challenges. The aetiology of pneumonia

often remains uncertain due to the inaccessibility of the lungs for sampling and the low sensitivity of current diagnostic tests for bacterial pneumonia (Jain et al., 2015; Nolan et al., 2018). Viruses are detected in the upper airway in the majority of children with radiographically confirmed pneumonia. However, many virus-positive children exhibit clinical features of bacterial infection requiring antibiotic treatment, despite the lack of microbiological evidence of bacterial involvement. Rhedin et al. reported a notably high cutoff of 430 µg/L among children with lower respiratory tract infections, for optimal discrimination between viral and bacterial aetiologies (Rhedin et al., 2022). This high cutoff may have been partly influenced by the limitations of current diagnostic tools for bacterial lower respiratory tract infections. In their study, only five out of 326 infected children were diagnosed with bacterial infections alone, while 20% had an undetermined aetiology. A similar challenge was observed in our Study II, where approximately one third of the population presented with lower respiratory tract infections.

Other factors that may influence the variation in cutoff levels across populations include the differing intensity of MxA responses to specific viruses, and the stronger MxA responses observed in febrile respiratory infections compared to non-febrile infections (Rhedin et al., 2022; Toivonen et al., 2015).

As with other infection biomarkers, no single definitive cutoff can be established for MxA, as there will always be a zone of uncertainty. For a viral biomarker, high specificity is essential to minimise the risk of undertreating a bacterial infection. In this context, a cutoff of 200 µg/L, which demonstrated 100% diagnostic specificity in Study III, appears suitable in clinical practice at the ED. A WHO international standard for MxA would aid in establishing reference ranges and ensuring comparability across methods and laboratories.

6.3.2 POC MxA

In Study III, POC MxA measurement showed minor to moderate difference (mean difference -76 µg/L) compared to the laboratory EIA reference method. At a 200 µg/L cutoff for antiviral response, 87% of POC MxA results aligned with the reference method, with 98% agreement for values above the cutoff. The analytical accuracy of the POC test was deemed adequate to confirm an antiviral response in the clinical context. Since POC values were slightly lower than reference values, a positive POC result provides stronger evidence of a viral infection. POC MxA's discriminative ability between viral and bacterial infections, excluding viral-bacterial coinfections, was excellent.

Timely access to reliable diagnostic tools is essential for optimising antibiotic use. Capillary blood sampling for POC testing offers advantages over traditional laboratory testing, including faster results, less invasiveness, and reduced resource

and training needs. POC testing for MxA provides results within < 15 minutes compared to several hours required for laboratory-based EIA methods. Unlike EIA, which faces delays due to limited laboratory availability and extended turnaround times, the POC MxA test is well-suited for ED use, providing actionable results.

Our findings are novel, as Study III was the first to evaluate a quantitative POC test for MxA measurement. Previous research on a combined qualitative POC test for MxA and CRP (FebriDx) has primarily focused on adult outpatient populations, using an MxA cutoff of 40 µg/L. However, this cutoff appears too low for children, as indicated by our results and those of other studies (Engelmann et al., 2015; Rhedin et al., 2022; Toivonen et al., 2015). Quantitative measurement offers a distinct advantage by improving test specificity at higher MxA values. It also enables defining individualised cutoffs tailored to the target population. Furthermore, the visually interpreted FebriDx test does not account for the possible presence of a coinfection. In cases where both MxA and CRP exceed their respective cutoffs, it interprets the result as indicating a viral infection. While studies on FebriDx suggest a minimal risk of coinfection (0–8%), this is notably lower than the rates observed in our population (Onrubia & González, 2020; Shapiro et al., 2022; Shirley, 2019).

6.3.3 MxA/CRP Ratio

Across Studies II and III, the MxA/CRP ratio demonstrated high diagnostic accuracy in the discrimination between viral and bacterial infections with AUCs of 0.89 and 0.97, respectively. This performance surpasses that of MxA alone, which is expected, since the MxA/CRP ratio integrates to complementary biomarkers: MxA, indicative of antiviral immune activation, and CRP, reflective of inflammation typically associated with bacterial infections. These findings are consistent with previous research (Engelmann et al., 2015; Iliopoulou et al., 2024; Metz et al., 2023). In our studies, the optimal cutoff for the MxA/CRP ratio ranged from 11 to 19. Previous studies have identified slightly lower cutoffs for adults (ranging from 2 to 5), but a value of 20 has been reported for children. In the clinical setting, a high MxA/CRP ratio indicates a viral infection, with a decreasing likelihood of bacterial coinfection as the ratio increases. Conversely, a low MxA/CRP ratio suggests a bacterial infection (low MxA, high CRP), a viral-bacterial coinfection (high MxA, high CRP), or a non-infectious disease (low MxA, low CRP).

However, it should be noted that in our study, the attending physicians who made the diagnoses had access to the routinely measured CRP results, which may have influenced the performance of the ratio.

6.3.4 Implications for MxA Use

MxA has potential as an adjunctive diagnostic tool, but its use should be tailored to specific clinical scenarios. The greatest utility of MxA measurement likely lies in the diagnostic evaluation of children, particularly infants, presenting with fever without a source. In these situations, rapidly confirming a viral infection through POC MxA measurement, particularly when combined with low PCT levels (or CRP, depending on the disease stage), could potentially reduce the need for further diagnostic procedures, unnecessary hospitalisations, and antibiotic use. *In vitro*, MxA expression is induced rapidly within 1–2 hours after IFN stimulation (Ronni et al., 1993). Similarly, therapeutic IFN-α or IFN-β induces MxA expression within a few hours (Gilli et al., 2006; Jorns et al., 2006). Thus, an antiviral response may be detected in the early stages of febrile illness, in some cases before localised symptoms become apparent. This is particularly relevant for febrile infants, who are typically brought to the ED promptly by their parents, with one study reporting a median duration of fever of 4 hours prior to arrival (Velasco et al., 2024). Given its slower kinetics, CRP typically remains within the normal range at this early stage, limiting its diagnostic utility. Furthermore, with the increasing use of multiplex respiratory virus PCR testing in EDs as part of the diagnostic workup, MxA could assist in determining the clinical significance of a detected virus, differentiating between a pathogenic infection and asymptomatic viral shedding.

A study evaluating the utility of FebriDx in children with acute respiratory infections at a Spanish ED reported that it influenced management in 10% of all children and 35% of those with pneumonia (de la Matta Farrando et al., 2024). While FebriDx appeared to reduce antibiotic prescriptions in some children with pneumonia, it did not result in an overall reduction in antibiotic use, as the test prompted antibiotic initiation in certain cases where it was not initially planned. Although this could partly reflect better antimicrobial targeting, it underscores the challenges of using biomarkers in treatment decisions, as they can both support and complicate clinical decision-making. Additionally, the authors suggested that FebriDx could help reduce the need for chest X-rays, noting that nearly half of the patients underwent imaging, yet fewer than one-third of the X-rays showed abnormal findings.

In the context of recent and potential future pandemics, MxA measurement could serve as a triage tool in crowded EDs. By quickly identifying and cohorting patients with viral infections, MxA testing might help prevent nosocomial infections and improve patient flow in high-pressure healthcare settings.

In addition to distinguishing between viral and bacterial infections, MxA may help determine whether a symptomatic child has an infectious disease at all, particularly when there are no signs of bacterial infection. This includes scenarios such as prolonged fever of unknown origin or gastrointestinal complaints, both of

which are common reasons for seeking care at the ED (Sands et al., 2012). In these situations, a low MxA level could point to a non-infectious aetiology, such as an immunological or functional condition. However, it is important to recognise that in cases of prolonged fever, MxA can also be induced in certain systemic rheumatologic conditions (Connor et al., 2006; Huijser et al., 2019; Maria et al., 2014; Soponkanaporn et al., 2019). To date, there are no data on MxA levels in Kawasaki disease, the most common paediatric vasculitis and an important consideration in the differential diagnosis of febrile children.

Potential barriers to integrating MxA POC test into the clinical workflow of a paediatric ED include the cost of the test, the need for personnel training and the time and effort required for sampling and analysis. However, since POC tests for CRP and WBC are already routinely used in the unit where our studies were conducted, implementing the MxA test alongside CRP measurement would be a logical addition. If the use of MxA POC test were to reduce the length of stay at the ED, additional diagnostic testing, antibiotic use, or hospitalisation, it could offer a cost-effective complement to existing diagnostic tools.

6.4 Gene Expression Profiling (IV)

Study IV highlighted both the potential and challenges of transcriptomics to differentiate between bacterial and viral infections in hospitalised children. While distinct blood transcriptome patterns were observed between healthy children and those with confirmed bacterial infections, the gene expression profile of children with confirmed viral infections was more heterogeneous. This heterogeneity likely reflects the varying aetiology, clinical presentations, and illness severity within this group, underscoring the complexity of applying transcriptomics in paediatric infectious diseases. Further stratification by infection type could enhance precision.

Despite these challenges, we identified a novel promising 2-transcript signature involving the *TSPO* and *SECISBP2* genes, capable of distinguishing bacterial and viral-bacterial coinfections from viral infections. This signature demonstrated good diagnostic accuracy (combined AUC 0.87) in both the discovery group (respiratory tract infections) and the validation group (non-respiratory infections), despite differences in clinical presentations. Remarkably, the signature outperformed CRP and PCT, even though these markers were partially used in the aetiologic classification.

The identification of translocator protein (TSPO) as a key component of the signature is biologically plausible, given its roles in mitochondrial function, immune regulation and inflammation (Batarseh & Papadopoulos, 2010; Betlazar et al., 2020; Papadopoulos et al., 2006). TSPO influences various cellular processes, including the production of reactive oxygen species, mitochondrial energy regulation, and

modulation of inflammatory responses. Its expression has been linked to bacterial infections and systemic inflammation, supporting its relevance in distinguishing bacterial from viral aetiologies (Blevins et al., 2021; Mahajan et al., 2016; Rupprecht et al., 2010; Wong et al., 2009).

For example, *TSPO* was identified among the 66 transcripts differentiating infants with SBIs from those without (Mahajan et al., 2016). It is also upregulated in children with sepsis or systemic inflammatory response syndrome (Wong et al., 2009). In contrast, in children with influenza, *TSPO* was among the top 50 underexpressed transcripts compared with bacterial infections (Herberg et al., 2013).

To our knowledge, no prior studies have examined *SECIS Binding Protein 2* (*SECISBP2*) expression in acute infections. SECISBP2 regulates the incorporation of selenium into selenoproteins, which have diverse antiviral, anti-inflammatory, antioxidant, and immunostimulatory effects (Avery & Hoffmann, 2018; Guillin et al., 2019; Rayman, 2012). Selenium reduces the incidence and severity of viral infections by enhancing both adaptive and innate immune responses (Bae & Kim, 2020). Selenium deficiency, in contrast, is linked to increased viral replication and worse clinical outcomes during viral infections (Beck et al., 2001, 1994; Moghaddam et al., 2020). The observed upregulation of *SECISBP2* may reflect selenium's antiviral effects mediated by selenoproteins.

Most previous RNA signature studies have focused on microbiologically confirmed single-aetiology cases, often excluding complex patient populations with probable or mixed aetiologies (Habgood-Coote et al., 2023; X. Hu et al., 2013; Ko et al., 2023; Ramilo et al., 2007; Rao et al., 2022; Ravichandran et al., 2021; Sweeney et al., 2016; Zaas et al., 2009). In studies with well-defined aetiologies, discriminative accuracy has been excellent, with AUCs exceeding 0.90, sensitivities of 87–100%, and specificities of 82–96% (Herberg et al., 2016; Mahajan et al., 2016; Schlapbach et al., 2024). However, performance has been less robust in probable infection cases or milder presentations (Barral-Arca et al., 2018; Schlapbach et al., 2024).

A few large multicentre studies have reported the performances of transcript signatures in complex phenotypes. In the U.S., a study validated a 45-transcript signature in adults and children over the age of 2 years with febrile RTI. Including cases without microbiological verification, the signature identified bacterial infections with a sensitivity of 86% and specificity of 72% (Ko et al., 2022). Another study validated an 87-transcript signature in adults and adolescents with RTI, classifying 82% of patients with probable bacterial infections and 71% with viral-bacterial coinfections as having either bacterial infections or coinfections, respectively (Lydon et al., 2019). Most recently, an Australian study reported an AUC of 0.86 in their validation group using a 10-transcript signature to differentiate both definite and probable bacterial infections from viral infections (Schlapbach et

al., 2024). The biosignature achieved AUCs of 0.76 and 0.81 for distinguishing definite or probable viral infections from coinfections, respectively. In our study, an AUC of 0.87, sensitivity of 77% and specificity of 87% for detecting bacterial infections in the combined discovery and validation groups align with these findings.

Interestingly, the 2-transcript signature by Herberg et al. performed poorly in our study (Herberg et al., 2016). This could be due to the high prevalence of viral-bacterial coinfections in our cohort, where interferon-stimulated genes like *IFI44L* failed to distinguish between viral infections and coinfections, as both elicit similar antiviral responses. Additionally, Herberg's signature was derived from children with severe, microbiologically confirmed infections, unlike our population, where only one-third had single-aetiology infections, and another third had coinfections. The discriminative accuracy of RNA signatures generally declines when differentiating among three or more phenotypes (bacterial, viral, non-infectious) compared to two.

Our RNA signature differentiated bacterial infections and viral-bacterial coinfections from viral infections, suggesting its potential for targeting antibiotic treatment. Its ability to identify viral-bacterial coinfections as bacterial is particularly advantageous in hospitalised children, where coinfections are prevalent. Unlike direct pathogen detection by PCR, gene expression analysis offers the advantage of measuring the antiviral host response, allowing it to distinguish between incidental and pathogenic viral findings and identify potential coexisting bacterial infections.

Although the identified 2-transcript signature was internally validated in our own dataset, the finding is novel and preliminary, and must be further investigated and externally validated in different clinical settings. Rapid results would be essential to impact diagnostic decision-making. Recent developments in POC tests for transcript signatures are promising in this regard (Ko et al., 2022; Pennisi et al., 2022; Tsalik et al., 2021). A potential advantage of our signature, compared to many others, is its minimal size (only 2 transcripts), which facilitates the development of a qPCR-based POC test.

6.5 Strengths and Limitations (I–IV)

A strength of studies II, III and IV, was the recruitment of a relatively large population of children with a wide age range, co-morbidities and diverse disease aetiologies. This diversity ensured that the populations closely reflected the real-world patient demographics in a paediatric hospital setting. Upon publication, Study II was the largest study of antiviral MxA responses in children, which had included coinfections in the analyses. Another advantage was the systematic use of respiratory virus PCR testing, enabling a detailed aetiologic classification. Importantly, in Study III, we evaluated what is, to our knowledge, the world’s first quantitative POC MxA

measurement directly at the point of care, at the paediatric ED, ensuring the results' clinical applicability.

As with most diagnostic performance studies, the absence of a golden reference standard for aetiologic classification of infectious diseases poses a limitation. Additionally, the diverse populations with varying infections introduced some uncertainty in aetiological classification. However, as noted above, this pragmatic study design enhances the generalisability of our results.

In Study I, the relatively small sample size of children with febrile UTI limits the broader applicability of our findings. In Studies II and IV, the number of children with a viral infection without bacterial infection was lower than anticipated among those with blood bacterial culture drawn. To address this, we included an additional sample of children with suspected viral infections. Moreover, in Study IV, healthy controls and a small subset of children with viral infections were recruited from a previous study conducted at a different time point.

In Study III, a limitation was that attending physicians mostly performed POC MxA tests by themselves, meaning they were not blinded to MxA results. However, as MxA testing is not routinely implemented in our unit and no specific interpretation on the results was provided, this is unlikely to have significantly influenced diagnostic decisions. Additionally, not all children in Study III underwent routine respiratory virus multiplex PCR testing. Consequently, the determination of aetiology relied primarily on clinical presentation rather than microbiological confirmation. Nonetheless, MxA demonstrated excellent discriminatory performance, even though some children classified as having a bacterial infection alone would have been virus-positive if universal virus testing had been used.

6.6 Future Perspectives

Further research is needed to evaluate the utility of MxA across diverse clinical scenarios. Key populations to study in greater depth are children presenting to the ED with fever without a source and those with lower respiratory tract infections, a leading cause of hospitalisation and a significant driver of antibiotic use in the paediatric population. Despite MxA's broad antiviral activity, its production during infections caused by less common viruses, such as different herpesviruses, is not well known and requires further investigation. Similarly, the behaviour of MxA in atypical bacterial respiratory infections remains unclear. In the study by Rhedin et al., only two cases of *Mycoplasma pneumoniae* were reported, both showing elevated MxA levels (> 350 µg/L) (Rhedin et al., 2022). Similarly, in our Study II, four children tested positive for either *Mycoplasma pneumoniae* or *Chlamydia pneumoniae*, with a median MxA level of 350 µg/L. However, two of these children were also positive for rhinovirus or human herpesvirus 7, both of which are

commonly found in asymptomatic children. As a result, definitive conclusions about the impact of atypical bacteria on MxA levels cannot yet be drawn. Most importantly, the next logical step toward clinical implementation would be a study evaluating the impact of MxA-guided treatment in paediatric EDs or primary care settings, focusing on its potential to reduce unnecessary diagnostic tests and antibiotic use.

Globally, the greatest potential benefit of POC MxA measurement would likely be achieved in low- and middle-income countries, where the burden of infectious diseases is highest, antibiotic misuse is widespread, and access to conventional laboratory testing is often limited. However, the use of MxA in these settings has been minimally studied. To date, only one study has reported the use of FebriDx in adults with acute febrile illness in an Ethiopian hospital ED. In that study, just 8% of patients had confirmed viral infections, all due to dengue fever, and the sensitivity of MxA for detecting dengue was low, only 27% (Akelew et al., 2020). This could be because dengue can block type I IFN-mediated signalling, thereby inhibiting the production of MxA (Aguirre et al., 2012). These findings highlight the potential of MxA to behave in contradictory or unexpected ways during infections caused by certain pathogens.

As an ideal marker does not exist, most current single-analyte infection biomarkers have imperfect sensitivity and specificity, performing well within defined clinical contexts. Given the superior performance of biomarker combinations over individual biomarkers, future research should explore MxA in conjunction with both currently used and emerging biomarkers, such as PCT and TRAIL. Leveraging machine learning algorithms to integrate clinical signs with MxA and other biomarkers could further improve diagnostic accuracy and enhance usability in clinical practice.

Transcriptomics research faces the challenge of balancing patient heterogeneity in discovery cohorts. While clinical homogeneity appears important for identifying robust biomarkers, biosignatures must remain broadly applicable to all febrile children, regardless of the underlying infection type. Achieving this requires careful study design to ensure both specificity and generalisability. Future studies should validate biosignatures composed of a limited number of transcripts for use in diverse patient populations and clinical settings. Further validation of the *TSPO* and *SECISBP2* signature identified in our study would be necessary to confirm its performance.

The evolution of microbiome analysis, particularly through advanced techniques such as mNGS, holds potential in significantly improving the diagnostic precision of infectious diseases. By enabling the simultaneous detection of a broad spectrum of pathogens, including fastidious and uncultivable ones, mNGS overcomes many limitations of conventional methods. Despite challenges in interpretation, cost and

accessibility, ongoing advancements in bioinformatics and sequencing technologies are likely to make both transcriptomics and microbiomics clinically accessible in the future.

For a diagnostic tool to significantly impact diagnostic decision-making and antibiotic use, its turnaround time is nearly as critical as its accuracy. In this regard, protein biomarkers (proteomics) offer advantages over transcriptomics or microbiomics due to the potential for simple, cost-effective POC test development. However, no single diagnostic test, or even a combination of tests, should be used in isolation, as clinical judgement remains the cornerstone of evaluating febrile children. That said, clinical judgement is subjective and heavily influenced by the physician's experience. In this context, biomarkers provide objective data, which may be particularly valuable for less-experienced physicians. By integrating biomarker-based diagnostics into clinical protocols, these tools have the potential to optimise antibiotic stewardship.

Finally, a major challenge in the diagnosis of paediatric infectious diseases lies in the striking heterogeneity of patients, infection sites, and aetiologies, and the occurrence of coinfections. Addressing this requires future research to adopt a standardised infection phenotyping and focus on developing biomarkers in the intended target population. Moreover, research efforts should reflect the complexity of real-world clinical settings by focusing on diagnostically challenging patients – those most likely to benefit from novel diagnostic approaches.

7 Conclusions

This thesis explored the diagnostic potential of blood MxA protein and host gene expression profiling in differentiating viral and bacterial infections in children.

In Study I, among children with febrile UTI, 40% tested positive for respiratory viruses, although only half of the virus-positive exhibited respiratory symptoms. MxA effectively distinguished virus-positive children with respiratory symptoms from virus-negative children without respiratory symptoms, reaching an AUC of 0.96. At a cutoff value of 409 µg/L, sensitivity was 100%, and specificity 91%.

Study II evaluated children hospitalised with suspected serious infections. MxA levels were significantly higher in viral infections and viral-bacterial coinfections compared to bacterial infections, even in cases of incidental virus detection. At a cutoff of 256 µg/L, MxA achieved an AUC of 0.81, with 74% sensitivity and 80% specificity in differentiating viral from bacterial infections.

Study III validated a novel quantitative POC MxA test that provides rapid results (< 15 minutes) with acceptable accuracy (mean difference: -76 µg/L), making it suitable for ED use. Among febrile children at the ED, MxA at a cutoff of 101 µg/L achieved an AUC of 0.96, with 92% sensitivity and 91% specificity in distinguishing viral from bacterial infections.

Study IV used host gene expression analysis to identify a novel 2-transcript biosignature (*TSPO* and *SECISBP2*) with promising diagnostic accuracy for differentiating bacterial and viral-bacterial coinfections from viral infections. The biosignature reached an AUC of 0.87, with 77% sensitivity and 87% specificity, and performed consistently across both the discovery cohort, which included children with respiratory infections, and the validation cohort, which consisted of children with non-respiratory infections.

In conclusion, our studies demonstrated that MxA is a highly effective biomarker for symptomatic viral infections, with measurement readily available through a POC test, supporting its integration into paediatric acute care. Considering the risk of viral-bacterial coinfections, combining MxA with a bacterial biomarker appears reasonable. Future research should assess the clinical impact of POC MxA testing in reducing unnecessary use of diagnostics and antibiotics.

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Once in Turku,
Ruut Piri

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