Turun yliopisto
University of Turku

DIAGNOSTIC STUDIES IN CHILDREN WITH ACUTE INFECTIONS:

Microbes and biomarkers

Lauri Ivaska

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
Sarja - ser. D osa - tom. 1269 | Medica - Odontologica | Turku 2017

University of Turku

Faculty of Medicine
Institute of Clinical Medicine
Department of Paediatrics
University of Turku Doctoral Programme of Clinical Investigation
Department of Paediatrics and Adolescent Medicine, Turku University Hospital
Turku, Finland

Supervised by

Professor Ville Peltola, MD, PhD
Department of Paediatrics and
Adolescent Medicine
University of Turku and Turku University Hospital
Turku, Finland

Reviewed by

Docent Tarja Heiskanen-Kosma, MD, PhD
Department of Pediatrics
University of Eastern Finland and Kuopio
University Hospital
Kuopio, Finland

Docent Janne Aittoniemi, MD, PhD
Department of Clinical Microbiology
Fimlab Laboratories
Tampere, Finland

Opponent

Professor Tobias Tenenbaum, MD
Pediatric Infectious Diseases and Pulmonology
University Children's Hospital Mannheim and
Heidelberg University
Mannheim, Germany

The originality of this thesis has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

ISBN 978-951-29-6730-8 (PRINT)
ISBN 978-951-29-6731-5 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Painosalama Oy - Turku, Finland 2017

To Unto, Valto and all other kids

ABSTRACT

Lauri Ivaska

Diagnostic studies in children with acute infections: Microbes and biomarkers

University of Turku, Faculty of Medicine, Institute of Clinical Medicine, Department of Paediatrics, University of Turku Doctoral Programme of Clinical Investigation; Department of Paediatrics and Adolescent Medicine, Turku University Hospital, Turku, Finland

Annales Universitatis Turkuensis, Medica-Odontologica, Turku, Finland, 2017

Clinical examination is the basis of evaluation of febrile children. Additional laboratory tests can be advantageous.

We did four distinct clinical studies, mainly in the emergency department (ED) setting, to pragmatically evaluate the accuracy, feasibility and characteristics of different diagnostic tests in children with acute infections, and documented the etiology of febrile pharyngitis.

The results of this study show that point-of-care tests (POCTs) for white blood cell count and C-reactive protein (CRP) were feasible and relatively accurate in comparison with standard laboratory methods. The occurrence of discrepant plasma procalcitonin (PCT) and CRP levels in acutely ill children was 29%. We confirmed an earlier finding that PCT rises more rapidly than CRP in bacteremic patients and made a new observation that PCT increases also in patients with acute diabetic ketoacidosis. In addition, a multianalyte antigen detection POCT for respiratory viruses was proven user-friendly and specific. It gave an overall sensitivity of 83% for influenza viruses, 89% for respiratory syncytial virus but only 25% for adenovirus in comparison with the polymerase chain reaction method. Finally, we found that febrile pharyngitis was caused solely by viruses in 59%, by viruses together with group A streptococcus (GAS) in 13% and solely by GAS in 10% of the children. Blood myxovirus resistance protein A (MxA) levels were increased in most of the pharyngitis patients with virus detection further supporting the causative role of viruses.

These results highlight the usefulness of POCTs in ED setting. In addition, we completed the etiological picture of febrile pharyngitis.

Keywords: point-of-care, white blood cell count, C-reactive protein, procalcitonin, antigen detection, respiratory viruses, pharyngitis, group A streptococcus, MxA protein

TIIVISTELMÄ

Lauri Ivaska

Tutkimuksia lasten äkillisten infektioiden diagnostiikasta: mikrobeja ja merkkiaineita

Turun yliopisto, lääketieteellinen tiedekunta, kliininen laitos, lastentautioppi, Turun yliopiston kliininen tohtoriohjelma; Lasten ja nuorten klinikka, Turun yliopistollinen keskussairaala, Turku, Suomi

Annales Universitatis Turkuensis, Medica-Odontologica, Turku, Finland, 2017

Kuumeisen lapsen lääketieteellinen arvio perustuu ensisijaisesti potilaan kliiniseen tutkimiseen. Arvion apuna voidaan tarvittaessa käyttää laboratoriotutkimuksia.

Teimme neljä erillistä kliinistä tutkimusta selvittääksemme tiettyjen laboratoriokokeiden käytettävyyttä, tarkkuutta ja ominaisuuksia äkillisiä infektioita sairastavien lapsien diagnostiikassa. Tutkimukset tehtiin käytännönläheisesti ja ne toteutettiin pääsääntöisesti päivystyspoliklinikalla. Lisäksi selvitimme kuumeisen nielutulehduksen aiheuttajat.

Tutkimuksen tulokset osoittavat, että valkosolujen ja C-reaktiivisen proteiinin (CRP) nopeat vieritestit olivat käytettävyydeltään hyviä ja suhteellisen tarkkoja laboratorion vakiomenetelmiin verrattuna. Totesimme myös, että 29 %:lla äkillisesti sairastuneista lapsista prokalsitoniinin (PCT) ja CRP:n pitoisuudet plasmassa ovat keskenään ristiriidassa. Vahvistimme samalla aiemmat havainnot siitä, että PCT-pitoisuus suurenee CRP:tä nopeammin baktereemisilla potilailla. Teimme uuden havainnon PCT:n noususta akuuttia ketoasidoosia sairastavilla lapsilla. Antigeenin osoitukseen perustuva monianalyyttinen hengitystievirusten pikatesti osoittautui käyttäjäystävälliseksi ja tarkaksi. Vertailussa nukleiinihapon osoitukseen perustuvaan menetelmään testin herkkyys influenssaviruksille oli 83 %, RS-virukselle 89 % mutta adenovirukselle vain 25 %. Nielutulehduksen aiheuttajaksi todettiin 59 %:lla lapsista virus, 13 %:lla virus ja A-ryhmän streptokokki (GAS) yhdessä ja 10 %:lla GAS yksinään. Veren myxovirus resistance protein A (MxA) -pitoisuus oli suurentunut suurimmalla osalla viruspositiivisista potilaista tukien ajatusta viruksesta kyseisen taudin aiheuttajana.

Tuloksemme korostavat vieritestien käyttökelpoisuutta päivystyspoliklinikalla. Lisäksi täydensimme tietämystä lasten nielutulehduksen etiologiasta.

Avainsanat: pikatestit, valkosolut, C-reaktiivinen proteiini, prokalsitoniini, antigeenin osoitus, hengitystievirukset, nielutulehdus, A-ryhmän streptokokki, MxA proteiini

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ABBREVIATIONS

ADB	antideoxyribonuclease B
AMR	antimicrobial resistance
ANC	absolute neutrophil count
ASO	antistreptolysin O
BAP	blood agar plate
cDNA	complementary DNA
CDR	clinical decision rules
CMV	cytomegalovirus
CNS	central nervous system
CRP	C-reactive protein
CSF	cerebrospinal fluid
DNA	deoxyribonucleic acid
DKA	diabetic ketoacidosis
EBV	Epstain-Barr virus
ED	emergency department
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EONS	early-onset neonatal sepsis
GAS	group A streptococcus
GCS	group C streptococcus
GGS	group G streptococcus
HD	hemodynamic
Hib	*Haemophilus influenzae* type B
hMPV	human metapneumovirus
ICU	intensive care unit
IF	immunofluorescence
Ig	immunoglobulin
IQR	interquartile range
IP-10	interferon gamma-induced protein-10
LONS	late-onset neonatal sepsis
LOS	length of stay

mRNA	messenger RNA
MxA	myxovirus resistance protein A
NAAT	nucleic acid amplification technique
NPS	nasopharyngeal swab
PCR	polymerase chain reaction
PCT	procalcitonin
PICU	pediatric intensive care unit
POC	point-of-care
POCT	point-of-care test
qPCR	quantitative PCR
RADT	rapid antigen detection test
RNA	ribonucleic acid
ROC	receiver operating characteristic
RSV	respiratory syncytial virus
RT-PCR	reverse transcription PCR
SBI	serious bacterial infection
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
UA	urinalysis
UTI	urinary tract infection
WBC	white blood cell count

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by the Roman numerals I-IV. Some previously unpublished data are also included.

I Ivaska Lauri*, Niemelä Jussi*, Leino Pia, Mertsola Jussi, Peltola Ville. Accuracy and feasibility of point-of-care white blood cell count and C-reactive protein measurements at the pediatric emergency department. *PLoS One*. 2015;10(6):e0129920.

II Ivaska Lauri, Elenius Varpu, Mononen Ilkka, Ruuskanen Olli, Peltola Ville. Discrepancies between plasma procalcitonin and C-reactive protein levels are common in acute illness. *Acta Paediatr*. 2016;105(5):508-513.

III Ivaska Lauri, Niemelä Jussi, Heikkinen Terho, Vuorinen Tytti, Peltola Ville. Identification of respiratory viruses with a novel point-of-care multianalyte antigen detection test in children with acute respiratory tract infection. *J Clin Virol*. 2013;57(2):136-140.

IV Ivaska Lauri, Niemelä Jussi, Lempainen Johanna, Österback Riikka, Waris Matti, Vuorinen Tytti, Hytönen Jukka, Rantakokko-Jalava Kaisu, Peltola Ville. Aetiology of febrile pharyngitis in children: Potential of myxovirus resistance protein A (MxA) as a biomarker of viral infection. *J Infect.* (2017) Accepted Manuscript, doi:10.1016/j.jinf.2017.01.002.

*Equal contribution

1 INTRODUCTION

Acute infection, often accompanied by fever, is one of the most common reasons for children to visit a physician. Because of the significant number of children with febrile illnesses presented to the emergency departments (EDs) or primary care practitioners, the most important clinical challenge is to identify the patients with serious bacterial infections to promptly initiate effective antimicrobial treatment. Another challenge is to spare patients with non-bacterial illnesses from unnecessary treatment with antibiotics. Furthermore, advancements in the development and data on the clinical benefits of specific antiviral agents have generated a demand for rapid virus diagnostics (Heinonen *et al.* 2010).

Clinical assessment of a child is still the most important single investigation that guides the physician's decision-making. However, after excluding the gravely ill and those with symptoms or signs suggestive to a milder or more specific condition, there are still many febrile children in whom further evaluation is needed. This diagnostic dilemma can be addressed by two different approaches that complement each other.

Biomarkers are medical signs, typically biological compounds such as proteins, carbohydrates or nucleic acids that can be measured accurately and reproducibly outside the patient (Strimbu and Tavel 2010). When assessing children with acute infections, the probable microbiological etiology can be derived indirectly by measuring the response of the host, for example, from a blood sample, in this thesis defined as a biomarker. The alternate approach is to show the pathogen, or parts of it, directly from a sample taken from the site of infection. Results from tests should be delivered reliably and rapidly at the bedside to be effective in clinical decision making. Thus, clinical studies concerning diagnostic tests should be carried out pragmatically at the site where the tests are planned to be used.

This study aimed to evaluate the clinical feasibility and analytical accuracy of point-of-care (POC) tests (POCTs) for both biomarkers [white blood cell count (WBC) and C-reactive protein (CRP)] and for antigen detection of multiple respiratory viruses in the pediatric ED. We wanted also to determine the significance of discordant plasma procalcitonin (PCT) and CRP results in children with acute illnesses. In addition, we documented the microbiological etiology of febrile pharyngitis in children and the possible role of virus-specific biomarkers in the clinical assessment of the illness.

2 REVIEW OF THE LITERATURE

2.1 Acute infections in children

2.1.1 Epidemiology

Fever is a principal reason for seeking care in 15% of visits to the ED by children and adolescents under 15 years (Niska *et al.* 2010). Respiratory tract infections, the most common cause of fever, are leading reasons for visiting a physician in this age group (Charles *et al.* 2004; Rautakorpi *et al.* 2009). Moreover, children under 5 years of age have every year on average from four to six respiratory tract infection episodes. The disease burden is highest in the age group of 1-2 years (Monto and Sullivan 1993; Craig *et al.* 2010; Byington *et al.* 2015; Toivonen *et al.* 2016). Most of the episodes are caused by viruses and to a lesser extent by other micro-organisms (Monto and Sullivan 1993). Respiratory viruses, particularly respiratory syncytial virus (RSV), human rhinovirus and influenza cause also a high burden of hospital admissions, morbidity and mortality (Miller *et al.* 2007; Louie *et al.* 2009; Nair *et al.* 2010; Antonova *et al.* 2012; Geoghegan *et al.* 2016).

Serious bacterial infection (SBI) is often defined as one of the following: bacteremic sepsis, bacterial meningitis, bacterial pneumonia, pyelonephritis, acute osteomyelitis/septic arthritis or deep soft tissue infection. Another close syndrome, invasive bacterial infection, is usually defined by positive bacterial culture in a normally sterile site (e.g. blood or cerebrospinal fluid).

The epidemiology of SBIs has changed markedly over the past decades. Introduction of universal vaccination against *Haemophilus influenzae* type B (Hib) and more recently against *Streptococcus pneumoniae* have decreased the rate of bacterial meningitis, pneumonia as well as occult bacteremia dramatically (Centers for Disease Control and Prevention (CDC) 1998; Waddle and Jhaveri 2009; Ladhani 2012; Palmu *et al.* 2015; Cioffredi and Jhaveri 2016; Jaakola *et al.* 2016). Fever without a source is, by definition, an acute illness in a well-appearing child in whom a specific cause of fever cannot be identified by history taking and physical examination (Baraff *et al.* 1993). The prevalence of urinary tract infection (UTI) in febrile children without an apparent source of infection is 3-8% (Hoberman *et al.* 1993; Shaw *et al.* 1998; Waddle and Jhaveri 2009; Watt *et al.* 2010), and it remains the most common SBI both in infants and in a vaccinated pediatric population.

Compared with older infants, age under 3 months and especially under 28-30 days increases the risk for SBI in febrile infants. The incidence of SBI in this age group has been estimated to be approximately 10% (Pantell *et al.* 2004; Biondi and

Byington 2015; Cioffredi and Jhaveri 2016). The mechanism that exposes neonates to infections is probably multifactorial due to the immaturity of both innate and adaptive immunological mechanisms and yet not fully understood. At least the impaired T-cell function in this age group has been suggested to play a role (Nonoyama *et al.* 1995).

2.1.2 Microbiological etiology

There are approximately 20 different respiratory viruses with hundreds of different virus types that cause respiratory infections. Rhinoviruses are the most frequently detected causative agents of the common cold in all age groups (Monto and Sullivan 1993; Makela *et al.* 1998; Ruohola *et al.* 2009; Ruuskanen *et al.* 2011; Monto *et al.* 2014). Respiratory viruses play a major role in the microbiological etiology and/or pathogenesis of community-acquired pneumonia, acute otitis media, acute sinusitis and acute pharyngitis. However, the above-mentioned infections can be also caused by bacteria, particularly *S. pneumoniae*, *H. influenzae, Moraxella catarrhalis* or *Streptococcus pyogenes* [group A streptococcus (GAS)], and therefore are often treated by antibiotics (Juvén *et al.* 2000; Ruohola *et al.* 2006, 2013; Pettigrew *et al.* 2011; Shulman *et al.* 2012; Nocon and Baroody 2014; Jain *et al.* 2015; Nokso-Koivisto *et al.* 2015; Sillanpää *et al.* 2016).

In addition to respiratory infections, the majority of the enteric infections and febrile illnesses without an apparent source are caused by viruses (Colvin *et al.* 2012; Chhabra *et al.* 2013; Liu *et al.* 2016). The most common etiologic agent of UTI in children is *Escherichia coli* causing 75-90% of the cases (Elder 2011; Sakran *et al.* 2015). *E. coli* together with coagulase-negative staphylococci, *Staphylococcus aureus* and *Streptococcus agalactiae* are the leading causes of bacteremia in infants, accompanied by *Streptococcus pneumoniae* in older children (Greenhow *et al.* 2014; Jaakola *et al.* 2016). Moreover, detection of a virus or viruses in a febrile infant does not exclude a concomitant serious bacterial infection but makes it less probable (Byington *et al.* 2004).

2.1.3 Antimicrobial usage

A major part of antimicrobial use occurs in an outpatient setting and the prescription rate is highest among children under 2 years (Rautakorpi *et al.* 2009). A report from Sweden documented that 87% of the antibiotic consumption in humans in 2015 occurred in outpatient care (‘SWEDRES/SWARM - Consumption of antibiotics and occurence of antibiotic resistance in Sweden’ 2016). In an American study, antibiotics were used against clinical guidelines in almost 50% of all antibiotic prescriptions (Fleming-Dutra *et al.* 2016). In-hospital antimicrobial

use in pediatric patients concentrates on the young children as well (Amadeo *et al.* 2010; Osowicki *et al.* 2014). Inappropriate use of antibiotics has several disadvantages, including promotion of antibiotic resistance and potentially harmful dysbiosis of normal microbiota (Saari *et al.* 2015; 'Antibiotic Resistance Threats in the United States, 2013 | Antibiotic/Antimicrobial Resistance | CDC' 2016; Korpela *et al.* 2016). The United States of America National Institutes of Health recently stated that antimicrobial resistance (AMR) must be addressed by a multifaceted strategy including biomedical research concerning, for instance, drug development and rapid diagnostics, and considerable behavioral changes in patients and prescribers (Marston *et al.* 2016).

2.2 Diagnostics

2.2.1 Clinical judgement rules

Several studies have focused on developing algorithms based on clinical evaluation of febrile children to better identify those at either high or low risk for serious bacterial infection. A systematic review by Van den Bruel et al. pointed out certain clinical findings as "the red flags" that should trigger a response in a physician. These findings were: cyanosis, poor peripheral perfusion and petechial rash and to some extent parental concern and clinicians' instinct (Van den Bruel *et al.* 2010). The same group identified clinicians' gut feeling as an important tool to better identify serious bacterial infections in febrile children who otherwise have clinically been assessed as having a non-severe illness (Van den Bruel *et al.* 2012).

In 1985, Dagan et al. published criteria, later known as the Rochester criteria, to identify previously healthy infants under 3 months of age hospitalized for suspected sepsis with low risk for serious bacterial infection. In this study, researchers combined clinical findings (no sign of ear, soft tissue or osteoarticular infection) with a normal result from urinalysis (UA), WBC 5-15 E9/L and band count < 1.5 E9/L, and found that only one of the 144 (0.7%) study infants without any of the risk factors had a serious infection (Dagan *et al.* 1985). The Rochester criteria was followed by a modified Boston criteria and Philadelphia criteria for infants older than 28 days. These criteria include results from white blood cell investigation of the cerebrospinal fluid sample, and they do not exclude preterm infants or infants with medical conditions (Baskin *et al.* 1992; Baker *et al.* 1993).

The importance of UA in the clinical evaluation of febrile children in addition to clinical assessment was highlighted in an Australian study evaluating UK National Institute for Health and Clinical Excellence (NICE) 2007 guidelines. These guidelines provide a traffic light system for the assessment and initial management

of young febrile children under 5 years of age (National Collaborating Centre for Women's and Children's Health (UK) 2013). In their study, traffic light criteria based on clinical parameters alone failed to identify 13.8% cases of serious bacterial infection of whom 68.8% had UTI (De *et al.* 2013).

2.2.2 Biomarkers

2.2.2.1 White blood cell count (WBC)

WBC is probably the most widely used diagnostic biomarker in febrile children without a source of infection. Use of WBC as a biomarker for bacterial infection is based on the mobilization of neutrophils from the bone marrow to the circulation and further transport to the site of infection in order to destroy microbes. As a result, increased numbers of white blood cells, dominantly neutrophils, can be quantified from a peripheral whole blood sample in 4-24 hours. Blood levels >15.0-25.0 E9/L for total WBC, >10.0 E9/L for absolute neutrophil count (ANC), >1.5 E9/L for band count and so-called "left shift" in total white blood cell count or immature-to-total polymorphonuclear cell ratio being ≥20%, are considered elevated and thus suggest bacterial infection. However, the low levels of total white blood cells (<4.0 E9/L) and/or neutrophils in whole blood (<1.0 E9/L) can represent their high consumption in sepsis and therefore suggest bacterial etiology for the illness (Peltola and Peltola 2007; Rintala and Saxén 2011).

In a Finnish study conducted before the national pneumococcal conjugate vaccine program, *E. coli* and especially *S. pneumoniae* accounted for the majority of bacteremic infections with marked elevation of total WBC level. It is worth to note that also a virus infection, especially caused by adenovirus can elevate WBC levels (Peltola *et al.* 2006). After the successful and comprehensive infant vaccination program against *S pneumoniae* and advancements in the development of other biomarkers, the clinical importance of routine WBC determination as a part of the evaluation of febrile children seems now less important. Literature from the past years conclude that WBC measurement and/or absolute neutrophil count are not very useful biomarkers either in ruling in or ruling out serious bacterial infections in children. It is not a very sensitive or specific test for detecting serious bacterial infections (Hsiao and Baker 2005; Van den Bruel *et al.* 2011; De *et al.* 2014). Low WBC or ANC and a high immature-to-total neutrophil ratio are still endorsed and have clinical utility in the setting of early-onset but not late-onset neonatal sepsis (EONS, LONS) (Hornik *et al.* 2012a, 2012b).

2.2.2.2 C-reactive protein (CRP)

It was characterized as early as in 1930 that patients with pneumococcal pneumonia had high concentrations of a certain protein, later called CRP, in their plasma that reacted with the pneumococcal C-polysaccharide. Already days after the patients had recovered from pneumococcal infection, plasma concentrations of CRP had decreased substantially (Tillett and Francis 1930).

Acute phase proteins, such as CRP, are produced in the liver as a consequence of proinflammatory cytokine-mediated increases in hepatic protein synthesis. These proteins are released into circulation as a result of infection, inflammation or other tissue damage. The normal range of CRP concentration in plasma is below 10 mg/L but during an acute inflammation process, the concentration increases more robustly and more rapidly than concentrations of other acute phase proteins. These features together with the relatively inexpensive price of the CRP measurement have made CRP a widely used biomarker especially for bacterial infection (Peltola and Peltola 2007; Rintala and Saxén 2011).

Most of the invasive bacterial infections increase plasma CRP levels substantially. Measuring CRP has an added benefit in differentiating between the viral and bacterial etiology of pneumonia or meningitis (Sormunen *et al.* 1999; Virkki *et al.* 2002). Optimal cutoff levels for diagnostic purposes have been debated and depend on whether aiming in ruling the invasive bacterial infection in or out. The most widely used cutoff values for ruling infections out are 20 mg/L or 40 mg/L (Putto, Ruuskanen, *et al.* 1986; Peltola and Jaakkola 1988; Van den Bruel *et al.* 2011). Using higher cutoff values, such as 80 mg/L, increases the specificity of CRP measurement for invasive bacterial infection but at the same time decreases the sensitivity (Van den Bruel *et al.* 2011).

The main disadvantage of CRP is its lack of specificity due to ambiguity between increased levels of CRP which could be indicative of bacterial infection. Inflammation other than of infectious origin, burns, major surgical operations, myocardial infarction, gout attack and certain viral infections are known to increase CRP levels (Putto, Meurman, *et al.* 1986; Clyne and Olshaker 1999; Gabay and Kushner 1999; Peltola and Peltola 2007; Rintala and Saxén 2011).

2.2.2.3 Procalcitonin (PCT)

PCT, a precursor of calcitonin hormone, is mainly produced in thyroid tissue in a healthy state. During a severe bacterial infection, PCT is intensively produced in parenchymal tissues around the body. The PCT synthesis starts early only 2-4 hours after the onset of a bacterial infection and peaks in 6-24 hours. The half-life

of the protein is approximately 24 hours, but it can be present in circulation for up to one week after the infection onset (Linscheid *et al.* 2004; Davies 2015).

Investigations for PCT use as a diagnostic biomarker for severe infections in children originates from 1993 when Assicot et al. published a study where they showed that children with invasive bacterial infections had high concentrations of PCT in their plasma (Assicot *et al.* 1993). Plasma procalcitonin concentrations are often elevated also in patients with malaria (Chiwakata *et al.* 2001; Lubell *et al.* 2015). During the 21st century, PCT has been studied extensively. Several different cutoffs for PCT have been used but the most widely used threshold is 0.5 µg/L (Van den Bruel *et al.* 2011; Gomez *et al.* 2012; Wacker *et al.* 2013; Cies and Chopra 2014; Mahajan *et al.* 2014; Pierce *et al.* 2014; Lautz *et al.* 2016). It has been suggested that PCT outperforms CRP in detecting invasive bacterial infections in children with fever without a source (Gomez *et al.* 2012; Yo *et al.* 2012). However, a prospective study from The Netherlands concluded that in patients with low (<20 mg/L) or high (≥100 mg/L) CRP, PCT did not offer additional prognostic value (Nijman *et al.* 2014).

For adults, elevated plasma/serum PCT has been adopted as a diagnostic criteria for sepsis (Dellinger *et al.* 2013). Pediatric studies in the intensive care unit (ICU) environment have found PCT to be useful in excluding bacterial infections in critically ill children (Cies and Chopra 2014; Lautz *et al.* 2016). Another indication for PCT measurement, especially in the ICU, is the evaluation of the antimicrobial treatment. In a recent review, Lam et al. suggested that PCT measurement could have great value in the discontinuation/de-escalation of antimicrobial treatment in critically ill adult patients (Lam *et al.* 2016).

There are several clinical conditions where plasma PCT level increases in the absence of bacterial infection. These limitations were reviewed by Reinhart et al. (Reinhart *et al.* 2012). Extreme HD stress, like cardiac arrest, increases circulating PCT levels substantially (Fries *et al.* 2003; Los Arcos *et al.* 2008; Annborn *et al.* 2013; Engel *et al.* 2013). Maybe the most important pediatric population associated with an unspecified increase in plasma PCT levels are the newborns. The physiologic elevation occurs during the first 48 hours of life and thus complicates the interpretation of PCT results regarding the diagnosis of EONS, a major cause of antibiotic use. However, a nomogram has been created to overcome these challenges (Chiesa *et al.* 1998; Monneret *et al.* 1998; Turner *et al.* 2006; van Herk *et al.* 2016). In addition, malnourishment limits the usefulness of protein biomarkers such as PCT (Page *et al.* 2014).

2.2.2.4 Myxovirus resistance protein A (MxA)

Myxovirus resistance protein A (MxA) is an intracellular guanosine triphosphatase with a broad antiviral activity. MxA is coded by the myxovirus resistance 1 gene (Mx1), which is expressed because of types I and III interferon production. The production of MxA was first related to influenza A virus infection and hence named accordingly (Lindenmann 1962; Horisberger *et al.* 1983; Ronni *et al.* 1995). Myxovirus resistance protein A plays a key role in innate antiviral defense against a number of viruses and has been proposed as a potential biomarker for viral infections (Halminen *et al.* 1997; Mitchell *et al.* 2013; Engelmann *et al.* 2015; Haller *et al.* 2015; Toivonen *et al.* 2015). Clinical evidence on the use of MxA as a biomarker for viral infection is still scarce, but it seems that blood MxA concentrations above 175-200 µg/L would best differentiate symptomatic virus infections from bacterial infections and from asymptomatic virus findings/healthy individuals (Engelmann *et al.* 2015; Toivonen *et al.* 2015). Earlier studies measured MxA levels from peripheral blood lymphocytes by flow cytometry (Halminen *et al.* 1997; Koskenvuo *et al.* 2006). More recent studies (Engelmann *et al.* 2015; Toivonen *et al.* 2015) used an enzyme immunoassay (EIA) method for measuring MxA concentration from lysed whole blood samples (Towbin *et al.* 1992; Vallittu *et al.* 2008).

In addition to infectious diseases, blood MxA measurement has other clinical applications as well. It has been established as a clinical marker for neutralizing antibody production in patients with multiple sclerosis (MS) treated with ß-interferon (Vallittu *et al.* 2008; Bertolotto *et al.* 2015). Type I interferon production seems to be involved with the pathogenicity of Sjögren's syndrome and lupus erythematosus, and elevated blood MxA level has been proposed as a tool for the diagnosis of these conditions as well (Feng *et al.* 2012; Maria *et al.* 2014).

2.2.2.5 Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of TNF-family, was first described in 1995 (Wiley *et al.* 1995). Functions of the protein are partly mediated by natural killer cells and involve programmed cell death (apoptosis) of various kinds of tumor or virus-infected cells (Wei *et al.* 2005). Recent research related to TRAIL has mainly focused on its potential as a therapeutic agent in cancer (Zhang *et al.* 1999; Nicholson 2000; Cretney *et al.* 2002). In addition to its anti-tumor functions, TRAIL is involved in infection-triggered host immune response regulation. The production of TRAIL is induced by interferon-alpha and interferon-gamma, which both play a key role in innate immune response against virus infection and therefore it has been proposed as a potential biomarker together with other biomarkers identify viral etiology in

patients with acute infections (Oved *et al.* 2015; Eden *et al.* 2016; van der Does *et al.* 2016). These studies suggest that the optimal TRAIL serum concentrations to differentiate between viral and non-viral/bacterial etiology would be 70-94 pg/mL.

2.2.2.6 Other infection biomarkers

A wide range of biomarkers has been studied in infectious diseases, especially for detection or monitoring of sepsis.

Blood lactate has proven to be associated with mortality in acutely unwell patients (Weil and Afifi 1970; Fine-Goulden and Durward 2014). In a general pediatric intensive care unit (PICU) set up, blood lactate level upon admission was significantly higher in non-survivors than in survivors (Morris *et al.* 2012). In another study, levels above 5 mmol/L upon PICU admission predicted mortality (Hatherill *et al.* 2003). Still, a study in adults shows that the higher the blood lactate levels are, the worse is the outcome (Nichol *et al.* 2010; Ingelfinger *et al.* 2014).

Clinical suspicion of neonatal sepsis accounts for a large amount of antibiotic usage in early childhood. Hence, biomarkers that could better identify neonates with true infections have been sought. Proinflammatory cytokines such as interleukin 6 (IL-6) and cell surface antigens such as CD64 have been studied for this purpose. Results are promising, but the markers are not widely in routine clinical use yet (Soni *et al.* 2013; Simonsen *et al.* 2014; Chiesa *et al.* 2015).

Several other biomarkers have been studied for these purposes: proadrenomedullin, pentraxin 3 (PTX3), a triggering receptor expressed on myeloid cells 1 (TREM-1), the soluble urokinase-type plasminogen activator receptor (suPAR), group 2 phospholipase A(2) and presepsin have been shown to identify adult patients with sepsis (Rintala and Saxén 2011; Reinhart *et al.* 2012; Uusitalo-Seppälä *et al.* 2012, 2013; Zhang, Hu, *et al.* 2015; Fan *et al.* 2016; ten Oever *et al.* 2016). In children, serum soluble ST2 has been proven to be useful in differentiating bacterial etiology in patients with systemic inflammatory response syndrome (SIRS) (Calò Carducci *et al.* 2014).

An alternative approach combines results from several different biomarkers. A lab-score combining CRP and PCT values with urine dipstick analysis performed well in detecting bacterial infections among children with fever without a source in two separate cohorts (Bressan *et al.* 2012; Nijman *et al.* 2014). However, investigators concluded that routine use of both, PCT and CRP, is of limited additional benefit. An Israeli group identified a host-protein signature by combining blood levels of three distinct biomarkers based on massive bioinformatic screening in a cohort of both pediatric (56%) and adult (44%) patients. These biomarkers were CRP, TRAIL and interferon gamma-induced protein-10 (IP-10). The signature

differentiated viral infections from viral-bacterial or sole bacterial infections with high accuracy (Oved *et al.* 2015). The same group validated their finding in a sub-study with ED patients only, and a Dutch group used TRAIL and IP-10 to identify patients with microbiologically confirmed viral infection with good accuracy (Eden *et al.* 2016; van der Does *et al.* 2016).

2.2.2.7 Gene expression profiling

Gene expression or transcriptional profiling utilizes the microbe/pathogen specific differences in host gene-expression generated by the cellular immune response. These so-called "biosignatures" in the messenger ribonucleic acid (mRNA) of peripheral blood leukocytes can be measured using microarray, quantitative polymerase chain reaction (qPCR) or ribonucleic acid (RNA) sequencing techniques (Mahajan *et al.* 2013; Mejias *et al.* 2014). In infectious diseases, focus has been in the differential diagnostics between bacterial and non-bacterial/viral infections.

Ramilo et al. published a proof of concept study in 2007 where they could discriminate the etiology of acute infections in children caused by influenza A virus, *E. coli*, *Staphylococcus aureus* or *S. pneumoniae* based on gene expression profiles (Ramilo *et al.* 2007). This group, another group from Duke University and more recently a London, UK-based collaborative have used a gene expression profiling method in diagnostics of acute infections (Mejias *et al.* 2014; Suarez *et al.* 2015; Herberg *et al.* 2016; Mahajan *et al.* 2016; Tsalik *et al.* 2016). Herberg et al. identified a 2-transcript profile with good discriminative accuracy between viral and bacterial infections in children (Herberg *et al.* 2016). At the same time, PECARN (The Pediatric Emergency Care Applied Research Network) group identified a 10-gene transcript signature that identified infants with bacterial infection among all febrile infants younger than 60 days at the ED setting (Mahajan *et al.* 2016).

2.2.3 Virus diagnostics

Virus culture has been the gold standard for diagnosing a virus infection. In recent decades, nucleic acid amplification techniques (NAATs) have become the new mainstay for diagnosing most of the virus infections due to enhanced sensitivity, sufficient robustness and methodological rapidity compared with virus culture (Weinberg *et al.* 2004). However, nucleic acid-based techniques have also their limitations concerning the interpretation of positive findings and higher expenses still necessitating the use of virus culture, serology and antigen detection as clinical diagnostic tools (Jartti *et al.* 2008; Jansen *et al.* 2011; Humphries and Miller 2014).

2.2.3.1 Virus culture

Viruses from patient samples are inoculated in cell lines and then identified preliminarily based on virus-specific cytopathic effect observed by light microscopy. Because different viruses replicate in various ways in different cell lines, it is crucial for a virus laboratory to be informed which viruses are sought for. The final identification of viruses is carried out mainly by immunological methods, such as immunofluorescence (IF) with virus-specific monoclonal antibodies.

Virus culture has distinct features compared with other diagnostic methods giving it both advantages and disadvantages. It is laborious and to many extents, a slow method but allows identification of viruses that were not sought at the first place. Furthermore, specimen type is not as strictly defined as it is for most of the tests based on direct detection of virus antigens or nucleic acids. Virus culture can be done from various kinds of swabs taken from blisters, nasopharynx, oropharynx or from stool, urine or other secretion samples. Also more invasive specimens such as bronchoalveolar lavage fluid or tissue samples are possible for virus culture. Most of the clinically meaningful viruses can be isolated and identified by means of virus culture. As for all virological diagnostic methods, also for virus culture adequate specimen collection, handling and transportation are of great importance (Lappalainen *et al.* 2011; Humphries and Miller 2014).

2.2.3.2 Antigen detection

Viral proteins of most commonly isolated viruses can be detected by immunologic methods directly from the patient samples. Antigen detection tests are generally well-standardized, more rapid and less sensitive than virus culture or tests based on NAAT. The most commonly used methods are based on IF assays or on enzyme-linked immunosorbent assays (ELISA). In IF assays, cells from a patient sample are first fixed and then fluorescein-labeled by antibodies after which the slide can be examined to observe typical fluorescence patterns.

In ELISA tube format or microtiter format, the virus antigens from patient sample are brought in soluble form and then captured on the surface of a solid phase coated with virus specific antibodies. Enzyme-labeled secondary antibodies are then added followed by detection of coloring by spectrophotometric methods. Thus, detection is more objective compared with techniques based on microscopy and can be automated. Tests are generally inexpensive and enable processing of large numbers of samples. Antigen detection is used widely for diagnostics of respiratory viruses and enteric viruses.

Many rapid antigen detection tests (RADTs) are based on an immunochromatographic, lateral flow technique in which a self-contained membrane is used as the solid support to capture the viral antigens. Enzyme-labeled antibodies then bind with viral antigens to produce antigen-antibody complexes, which in turn are detected by the reaction of a chromogenic substrate and read visually. Results from lateral flow tests can be biased in interpretation and their performance is exposed to a variety of disturbances concerning for example the circulating virus type (Weinberg *et al.* 2005). They perform best during epidemics or during high seasons of the virus concerned (Lappalainen *et al.* 2011; Humphries and Miller 2014).

2.2.3.3 Nucleic acid detection

NAATs are mainly based on the enzymatic amplification and specified detection of the amplified target molecules by polymerase chain reaction (PCR). PCR relies on thermal cycling of the sample in the presence of specified primers, probes and enzymes resulting in exponential replication of the target nucleic acid. However, also isothermal NAATs are becoming more widely used, especially in point-of-care platforms (Buchan and Ledeboer 2014). Nucleic acids are first extracted from the sample. Then nucleic acids from RNA viruses are reverse transcribed to a copy of complementary deoxyribonucleic acid (cDNA) before the amplification (Lappalainen *et al.* 2011; Humphries and Miller 2014). The amplification product, the amplicon, can be detected by various methods such as gel electrophoresis, hybridization or by sequencing. In routine diagnostics, the amplified deoxyribonucleic acid (DNA) is detected in real-time without a separate detection step.

Because of the high sensitivity of NAAT methods, the potential for false positive results may increase and further complicate the interpretation of positive results. However, improvements in the automation of specimen processing has reduced the opportunity for amplicon contamination. Risk for contamination exists and must be considered also during sample collection.

In clinical work, NAATs are the mainstay for diagnosing viral central nervous system (CNS) infections and increasingly also for diagnosing acute respiratory infections using nasopharyngeal swab (NPS) samples or gastrointestinal virus infections from stool samples. Tests can be divided into single-analyte nucleic acid amplification tests, multiplexed assays for simultaneous detection of different viruses and viral nucleic acid quantitation assays. In addition, sequencing of the viral genome can be used for molecular typing of viruses, for epidemiological studies and transmission pattern investigations (Wohl *et al.* 2015).

Single-analyte NAAT assays and multiplexed assays can be either commercial or laboratory developed. In CNS infections, a PCR assay for herpes simplex virus from cerebrospinal fluid (CSF) has proven to be particularly useful in clinical decision making, including the discontinuation of empirical acyclovir treatment following a negative result (Lakeman *et al.* 1995; Caviness *et al.* 2008; Humphries and Miller 2014). Commercial multiplexed NAAT assays can be designed to detect from two up to eighteen respiratory viruses. Latest multiplexed NAAT assays for respiratory infections cover the majority of potential pathogens and thereby offer a diagnostic tool with potential also for unexpected and multiple virus detection. Multiplexed assays for detection of enteric viruses together with bacteria are also available (Jiang *et al.* 2014; Onori *et al.* 2014; Zhang, Niu, *et al.* 2015).

Clinical applications of quantitative NAATs include prediction of disease progression, differentiating between symptomatic and asymptomatic virus detection and monitoring the efficacy of antiviral therapy or potential resistance for antivirals. Quantitative measurement of viral load from blood is used for hepatitis B and C viruses, human immunodeficiency virus, cytomegalovirus (CMV), Epstain-Barr virus (EBV) and polyomaviruses (BK and JC viruses) (Humphries and Miller 2014). In addition, semi-quantitative information from tests based on NAAT, for example, cycle-threshold in PCR can be used to estimate the number of viruses in a sample: a low number of reaction cycles in PCR before a positive result indicates a high copy number of viruses in the sample.

2.2.3.4 Serology

Virus infections can be diagnosed by serological methods based on virus-specific immunoglobulin (Ig) G and/or IgM antibody measurements from a blood sample. The concentration of IgM antibodies in sera increases rapidly after the disease onset and decreases below the detection limit typically in 1-3 months, whereas IgG antibodies persist even for the rest of the individual's lifespan. As a sign of an acute infection, the serum level of IgG antibodies rises considerably between acute and convalescent samples. Another way to diagnose an active infection is to detect IgM antibodies from the acute serum sample. However, all virus infections do not trigger IgM antibody production and occasionally, serum IgM antibody levels may be misleading a long time after the infection onset. A third way for a serological diagnosis of a recent infection is to demonstrate low IgG avidity, in other words, the strength with which antibodies bind to their antigens (Enders *et al.* 2008; Lappalainen *et al.* 2011; Prince and Lapé-Nixon 2014).

Serological methods can be used in the diagnosis of a variety of virus infections. However, at its best, virus serology can identify a primary infection within weeks to months after symptom onset in an immunocompetent host. In contrast,

serological responses are not that useful in the diagnostics of virus infections in an immunocompromised host, in secondary infections (re-infections or re-activations) or in focal infections without systemic manifestations (Lappalainen *et al.* 2011).

Time from the symptom onset to the seropositivity depends on the virus. For example, nephropathia epidemica caused by Puumala virus from the genus *Hantavirus* leads to IgM antibody production during the first week of illness in practically all infected patients (Mustonen *et al.* 2013). For respiratory viruses, serological diagnosis is mainly based on documenting the increase in serum IgG antibody levels in two consecutive blood samples taken within a two-week interval. For viruses like the human rhinovirus with hundreds of different genotypes, clinically useful serological assays do not exist.

Table 1. Summary of distinct qualities of current virus diagnostic methods.

Method	***Virus culture***	***Antigen detection***	***Nucleic acid amplification***	***Serology***
Advantages	Open ended, relatively sensitive, applicable to different sample materials, potential for research and surveillance	Rapid, cheap, specific	Sensitive, specific, relatively rapid, comprehensive syndrome based multiplex assays	Easy sampling, possible to detect infection afterwards
Disadvantages	Laborious, slow	Less sensitive than NAAT, lack of tests for antigenically diverse viruses	Expensive, occasionally complicated interpretation of the significance of positive results	Lack of sensitivity and specificity, need for repeated sampling

NAAT, nucleic acid amplification technique

2.2.4 Diagnostics of group A streptococcus (GAS) pharyngitis

Throat infections caused by GAS can be diagnosed by several different methods. For this purpose, clinical decision rules (CDR) have been developed. Methods mainly used in the clinical setting are all based on sample collection by swabbing the oropharynx and include throat culture (golden standard), RADTs and increasingly also NAAT. Serological methods have relevance in the diagnostics of GAS pharyngitis only in research use.

2.2.4.1 Clinical decision rules

The Centor score for identification of GAS etiology of sore throat in adults was first introduced in 1981 (Centor *et al.* 1981) and later modified by age criteria to the McIsaac score (McIsaac *et al.* 1998). Features that point to GAS etiology include fever >38°C, absence of cough, anterior cervical adenitis and tonsillopharyngeal inflammation with exudates. In the original study (Centor *et al.* 1981), adult patients who had sore throat and all four criteria positive had a 56% chance of a GAS positive throat culture. A meta-analysis that assessed the efficacy of CDRs suggested that no clinical features are specific enough for diagnosing GAS etiology in children, but they can be used to target the patients who most potentially would benefit further diagnostic testing (Le Marechal *et al.* 2013).

Table 2. McIsaac score for patients with sore throat. Total score of ≥ 2 supports GAS testing. Modified from McIsaac *et al.* 1998 and used with the permission of Canadian Medical Association.

Criteria	*Point*
Temperature > 38°C	1
No cough	1
Tender anterior cervical adenopathy	1
Tonsillar swelling or exudate	1
Age 3-14 years	1
Age 15-44 years	0
Age > 45 years	-1

2.2.4.2 Throat culture

Throat culture on sheep blood agar plate (BAP) was first described in 1960 and has been accepted as the diagnostic standard for streptococcal pharyngitis (BREESE *et al.* 1960). A throat sample is taken by a swab from the tonsils/oropharynx ideally without touching other mucosa of the mouth or tongue. It is then inoculated on streptococcal-selective blood agar. Colonies of beta-hemolytic streptococci are then identified and serotyped. In addition, streptococcal selective broth can be used before the culture to enhance the sensitivity of the method.

In most of the clinical studies concerning epidemiology or treatment of GAS pharyngitis, the diagnosis is based on throat culture (Evans and Dick 1964; Glezen *et al.* 1967; Moffet *et al.* 1968; Kaplan *et al.* 1971; Douglas *et al.* 1984; Krober *et al.* 1985; McMillan *et al.* 1986; Putto 1987; Zwart *et al.* 2000; Hsiao *et al.* 2006; Danchin *et al.* 2007). As a result, also the guidelines and review articles concerning acute pharyngitis use throat culture on a BAP as the gold standard for GAS diagnosis (Bisno 2001; Pelucchi *et al.* 2012; Shulman *et al.* 2012; Blomberg *et al.* 2013). GAS is always sensitive to penicillin, and in this respect, microbiological diagnosis with throat culture including antibiotic susceptibility testing is not obligatory.

Sample transportation, incubation and streptococci identification takes typically 24-72 hours in a microbiological laboratory. To shorten this turnaround time, office-based culture methods on a streptococcal selective BAP are used. An American study compared the performance of RADT and a pediatric office-based culture with a standard throat culture in a laboratory. They found that the office-based culture gave a sensitivity of 81% and specificity of 97% compared with the laboratory culture values (Tanz *et al.* 2009).

2.2.4.3 Rapid antigen detection

Most of the present RADTs for GAS are lateral flow and immunochromatographic assays based on the detection of cell wall carbohydrates or other GAS-specific antigens. These tests provide qualitative results typically in approximately 15-20 minutes. A meta-analysis published in 2014 analyzed outcomes of 60 separate studies and concluded that GAS rapid tests offer varying sensitivity (summary estimate 86%, range 53-96%) with less variation in specificity (summary estimate 96%, only individual studies with specificity <90%). Best performed tests that were based on molecular techniques such as DNA probes, PCR and fluorescence in situ hybridization methods provided summary estimates for a sensitivity and specificity of 93% and 99%, respectively (Lean *et al.* 2014).

American guidelines recommend a systematical backup culture for children with a negative test result from GAS RADT unlike European guidelines that estimate the performance of RADT to be clinically satisfactory (Pelucchi *et al.* 2012; Shulman *et al.* 2012). There has been a debate concerning the necessity of backup culture (Dingle *et al.* 2014; Matthys 2015), and it has been suggested that confirming GAS RADT results by throat culture would not be cost effective (Lean *et al.* 2014). In addition, a novel highly sensitive antigen detection test reported pharyngeal GAS prevalence rates of approximately 200% in symptomatic patients compared with standard culture on BAP (Vakkila *et al.* 2015). The relevance of these findings remains unclear.

2.2.4.4 Nucleic acid detection

New test platforms based on the detection of DNA that are specific to GAS are being used with increasing frequency. Both, isothermal and real-time PCR assays exist delivering results generally in 1-3 hours, while some assays have turnaround times of 8 minutes or less (Cohen *et al.* 2015). Most of the assays seem to be more sensitive than RADTs when compared with throat culture (Felsenstein *et al.* 2014; Lean *et al.* 2014; Cohen *et al.* 2015). The high sensitivity might lead to a moderate increase in false positive results in comparison with standard culture. Some of these “false positive” results have shown to be positive for GAS also with

laboratory developed PCR assays (Felsenstein *et al.* 2014; Cohen *et al.* 2015). It remains uncertain if the higher sensitivity of NAAT assays is clinically relevant in terms of identifying more patients who would benefit from antibiotic treatment.

2.2.4.5 Serology

There are two main serological assays for the detection of antibodies produced against GAS: antistreptolysin O (ASO) antibodies and antideoxyribonuclease B (ADB) antibodies. Antistreptolysin O titer was originally measured by rather laborious dilutional assays. Today, it is replaced in routine use by latex agglutination or more recently nephelometric assays (based on measuring the turbidity of liquid sample by light that goes through it at an angle). An increase in serum ASO levels is not specific to GAS infections because Lancefield group C and G streptococci also produce streptolysin O. ADB antibodies are not that well-standardized. However, they might be more important in GAS skin and soft tissue infections than in pharyngitis and more specific to GAS than ASO antibodies (Steer *et al.* 2015).

It has been suggested that serum ASO level peaks in 3-5 weeks after infection, serum ADB level peak in 6-8 weeks and that the best evidence of an acute GAS infection is a titer increase from acute to convalescent serum samples (2-4 weeks apart) (Steer *et al.* 2015). In an earlier study on the diagnosis of GAS pharyngitis, ASO titer increase occurred in half of the symptomatic patients with a positive throat culture (Kaplan *et al.* 1971). An Australian family-study showed that in unequivocal primary GAS pharyngitis cases, the ASO titer increased in 32% and ADB titer in 21% in paired sera (Danchin *et al.* 2007).

2.2.5 Point-of-care (POC) testing

2.2.5.1 Definition and motivation

POCTs or bedside/rapid tests can be defined in many ways. By the most popular definition, they can be performed at the site of patient care, they do not need laboratory conditions, they can be operated by staff without laboratory education, they should be easy to use and they are often rapid enough to have immediate effect on the clinical decision making (Drancourt *et al.* 2016).

Motivation for POCT use depends on the context. In resource-limited settings, POCTs can be the only way to have access to any laboratory tests (Peeling and Mabey 2010). In the developed world, the main driver for POCT usage is the ability for timely results allowing impact on treatment decisions and hastened patient-flow. Point-of-care testing can potentially also reduce expenses (Kokko *et*

al. 2014). Moreover, POCTs suit well small-scale doctor's offices that operate without more advanced laboratory facilities.

2.2.5.2 Applications in pediatric infectious diseases

Diagnostic POCTs relevant to pediatric infectious diseases can be roughly divided in tests targeting direct pathogen detection and in non-pathogen-specific tests.

Pathogens that have been widely sought by rapid antigen detection tests include influenza viruses, RSV, to some extent adenovirus and GAS. An antigen test for *S. pneumoniae* has value in microbiological evaluation of pleural empyema but not as a urinary antigen test in the diagnosis of pneumonia in children (Neuman and Harper 2003; Navarro *et al.* 2004; Le Monnier *et al.* 2006; Ploton *et al.* 2006). Use of NAATs in POC diagnostics is increasing rapidly. Several rapid NAAT assays for influenza and RSV already exist and are followed by multiplexed assays with a syndromic approach. Causative agents of respiratory infection, enteric infection, CNS infection or bacteremic infection can already be sought by highly multiplexed NAAT that deliver results in 1-2 hours (Andersson *et al.* 2014; Humphries and Miller 2014; Drancourt *et al.* 2016). Parasites causing malaria and other sources of tropical fever such as dengue or, more recently, Ebola virus can be detected by POCTs based on either antigen detection or NAAT (Drancourt *et al.* 2016). Several well-performing rapid tests for detection of human immunodeficiency virus antibodies also exist including applications designed to use cell phones as their source of energy (Drain *et al.* 2014; Laksanasopin *et al.* 2015).

Non-pathogen-specific rapid tests are also routinely used in the diagnostics of acute infections in children. Presence of leukocyte esterase and/or nitrite in urine dipstick analysis can be used for the evaluation of a child with possible UTI. Especially *E. coli* but also other pathogenic bacteria cause pyuria and/or nitrate production in urine (Wald 2014; Shaikh *et al.* 2016). Blood levels of biomarkers indicative for severe bacterial infection can be determined by POCTs. Commercial assays are available at least for WBC and CRP, and in some form also for PCT. However, accuracy of the tests in distinct clinical settings are not widely evaluated and might have variation between different products (Esposito *et al.* 2005; Monteny *et al.* 2006; Osei-Bimpong *et al.* 2009; Verbakel *et al.* 2014; Kutz *et al.* 2016).

2.2.5.3 Studies on clinical impact

In developed health care systems, studies on the clinical impact of POCTs are focused on three key issues: impact on antimicrobial usage, impact on cost-effectiveness and patient-flow and impact on additional testing. Expectations for POC tests as a tool against AMR are especially high (Marston *et al.* 2016).

The impact of POCTs on antimicrobial use has been studied mainly in primary care setting and for respiratory tract infections. A Cochrane review concluded that use of POCTs such as CRP test by primary care physicians can reduce antibiotic use. However, depending on how the tests are used, they might also increase hospital admission rates (Aabenhus *et al.* 2014). Another review on PCT testing reported decreased antibiotic consumption as well but raised questions concerning their cost-effectiveness (Schuetz *et al.* 2012). A Canadian study found that rapid viral testing of febrile children in the emergency department significantly decreased antibiotic prescription rate after discharge. The same study did not find a statistically significant decrease in the ED length of stay (LOS) (Doan *et al.* 2009). A recent study from Netherlands reported that use of POC CRP test reduced the LOS at the ED significantly in comparison with traditional CRP testing (Nijman *et al.* 2015). A retrospective Finnish study found that use of POC testing for WBC and CRP reduced both LOS at the ED and laboratory costs (Kokko *et al.* 2014). The impact of rapid viral testing on the use of additional investigations is controversial. However, rapid viral testing decreases the use of additional chest X-ray examinations (Bonner *et al.* 2003; Doan *et al.* 2014).

3 AIMS OF THE STUDY

The objectives of this thesis were:

1. To evaluate the accuracy and clinical feasibility of POC WBC and CRP measurement at the pediatric ED compared with routine laboratory methods (I)
2. To determine the occurrence and clinical significance of discrepant plasma PCT and CRP levels in acutely ill children (II)
3. To compare the diagnostic performance of mariPOC® POC multianalyte respiratory virus antigen detection test performed at the pediatric ED with PCR test performed in laboratory conditions (III)
4. To document the microbiological etiology of febrile pharyngitis in children and adolescents in an outpatient setting, and to demonstrate the significance of virus detection by simultaneous measurement of blood MxA level (IV)

4 MATERIALS AND METHODS

More detailed description of materials and methods are presented in the original publications.

4.1 Subjects, study designs and data collection

The thesis is comprised of four original studies with four separate study populations.

Turku University Hospital

All patients in the Studies I-IV were treated in the Turku University Hospital, Turku, Finland. The Department of Pediatrics and Adolescent Medicine in Turku University Hospital serves a population of 70 000 children and adolescents 0 to 16 years. In addition, it serves as a level III hospital for two level II hospitals. The pediatric unit has 70 beds. The Department of Pediatrics and Adolescent Medicine has an ED of its own mainly serving as a referral clinic. The Department of Emergency Services serves as a walk-in-clinic for both adult and pediatric patients.

Patients in the clinical part of Study I and patients in Study III were all treated in the ED of the Department of Pediatrics and Adolescent Medicine. In Study II all patients were treated in the Department of Pediatrics and Adolescent Medicine. In this study, also admitted patients from the pediatric wards were included. For Study IV, all patients were recruited in the Department of Emergency Services. Data on study settings of the different parts of the thesis is summarized in **Table 3**.

Table 3. A summary of the study settings in original studies (I-IV).

Original study	*Short title*	*Study location/setting*	*Study period*	*Number of subjects/cases in the analysis*
I	WBC and CRP POC testing	Central laboratory	2008	77 (WBC), 48 (CRP)
		Pediatric ED	2008-2010, 2011-2013	171
II	Discrepancies in plasma PCT and CRP levels	Department of Pediatrics and Adolescent Medicine	2010	635
III	Multianalyte antigen detection POC test for respiratory viruses	Pediatric ED	2011	158
IV	Etiology of febrile pharyngitis in children	General ED	2013-2015	83

WBC, white blood cell count; CRP, C-reactive protein; POC, point-of-care; ED, emergency department; PCT, procalcitonin

4.1.1 Study on WBC and CRP POC testing (I)

Study I was done in the Central Laboratory and in the ED of the Department of Pediatrics and Adolescent Medicine of Turku University Hospital between 2008 and 2013.

In the first part of the study, WBC and CRP POC tests were validated for clinical use. WBC and CRP levels were measured in random clinical blood samples in parallel by POC and by reference methods in the hospital central laboratory. The 77 whole blood samples for the WBC and 48 plasma samples for the CRP measurements were originally taken for other analytical purposes.

The white blood cell count and the CRP POC tests were then adopted for clinical use in the pediatric ED. Testing was performed by the attending physicians who all were trained according to the test manufacturers' guidelines. In daily work in the ED, the attending physician used the POC tests when clinically justified. The blood sample for POC testing was collected by pricking the fingertip or heel of infants less than 3 months of age in the patient examination room. After the POC testing, venous blood samples were taken for laboratory measurement of WBC and CRP levels when it was considered clinically necessary.

In the second part of the study, pediatric ED patients who were tested by both, the POC and routine methods within the maximum 4-hour sampling interval, were identified retrospectively from the hospital patient records. The diagnosis and clinical course of these patients was retrieved, and the analytical accuracy of the POC tests were evaluated in comparison with the routine laboratory methods.

4.1.2 Study on PCT and CRP discrepancies (II)

For Study II, data was collected retrospectively. All patients who were treated in the Department of Pediatrics and Adolescent Medicine and had their plasma PCT and CRP levels determined simultaneously in the Central Laboratory between 1st of January, 2010 and 31st of December, 2010 were first identified from the laboratory data base. Then, serial measurements were excluded so that if the same patient had been tested repeatedly for PCT and CRP, only the first pair of samples was included in the study and referred as an individual case. If same patient had several distinct illness episodes during the study period, only the clinically separate episodes occurring at least 2 weeks apart were referred as individual cases. Data on the clinical course of these patients was retrieved from the hospital electronic records.

All cases were further categorized in four different groups based on the plasma PCT and CRP levels: PCT high, CRP high; PCT high, CRP low; PCT low, CRP high; and PCT low, CRP low. Plasma PCT concentrations ≥ 0.5µg/L and CRP concentrations ≥ 40 mg/L were considered elevated. Cutoff values were determined based on the current practices in our department and on the existing reports in children.

4.1.3 Study on multianalyte antigen detection POC test for respiratory viruses (III)

Study III was a prospective study done at the pediatric ED between 11th of January and 17th of March, 2011. The time period was chosen based on the typical peak season in respiratory infections during which the need for rapid diagnostic testing of respiratory viruses appears to be the highest. Patients in the ED were eligible for the study if they were admitted to the hospital with respiratory symptoms or if they were treated as outpatients and the attending physician suspected an infection caused by influenza viruses, RSV or adenovirus. These groups of patients were selected because they are routinely tested for respiratory viruses in our unit. Families of all patients filled a standardized inquiry about the child's chronic medical conditions, about the duration of specific symptoms and about influenza immunization status.

A NPS sample was collected using a flocked swab (Copan, Brescia, Italy). The swabs were eluted in buffer after which mariPOC® (ArcDia International Oy Ltd., Turku, Finland) test was performed at the ED according to manufacturer's instructions. ED nurses were first trained (1 h training) to use the test system and then took the samples and performed the testing. The remaining sample solution was transferred to the Department of Clinical Virology where reference PCR-tests were performed immediately for most of the admitted patients. In 11 of the

admitted patients, a new NPS sample for respiratory virus PCR was taken, analyzed in 72 hours from POC testing and used as a reference test. Samples from outpatients were frozen in -70°C until analyzed by PCR.

4.1.4 Study on the etiology of febrile pharyngitis

Study IV was done in The Department of Emergency Services at Turku University Hospital between 25th of November, 2103 and 31st of January, 2015. It was a prospective observational cohort study addressing the etiology and diagnostics of febrile pharyngitis. Children and adolescents 1 year to 16 years old with acute pharyngitis were eligible for the study. The inclusion criteria were introduced to the ED physicians who performed the recruitment. Criteria for acute pharyngitis were fever (body temperature ≥ 38.0°C at the ED or reported by parents during the current illness) and exudates or intense redness in the oropharynx. At the enrollment, patient's symptoms, clinical findings and underlying conditions were recorded, and throat swab samples and blood samples were collected. Each family received a symptom diary card and was contacted by telephone after the enrollment visit to record the duration of patient's symptoms. All patients were invited for a control visit minimum 10 days after the enrollment. At the control visit, symptoms and clinical findings were recorded, and throat swabs and blood samples were obtained.

During the enrollment visit, four throat swabs were collected by rubbing the swabs against both tonsils being: 1) StrepTop® (All.Diag, Strasbourg, France) an immunochromatographic GAS rapid test that was performed by the staff nurses at the ED according to manufacturer's instructions, 2) a flocked swab for mariPOC® (ArcDia, Turku, Finland) automated immunometric GAS rapid test that was sent to and analyzed at the on-call-laboratory, 3) an eSwab® sample that was sent for bacterial culture to the laboratory of clinical microbiology and 4) one flocked swab (Copan Brescia, Italy) that was sent for virus PCR testing to the Laboratory of Clinical Virology. Results from GAS rapid tests are not reported in this study. Venous blood samples for biomarker measurements were taken by laboratory nurses in the emergency department during the enrollment visit.

4.2 Measuring biomarkers in blood (I, II, IV)

In Study I, rapid testing was performed by HemoCue WBC® (HemoCue AB, Ängelholm, Sweden) and by Afinion AS100 CRP (Axis-Shield PoC AS, Oslo, Norway) POC analyzers. The reference methods were laboratory-based WBC (Sysmex XE-2100 analyzer; Sysmex, Kobe, Japan) and CRP (Modular P, Roche Diagnostics, Mannheim, Germany) measurements.

The HemoCue WBC analyzer is a WBC POC test system that rests on microcuvette technology. The analysis requires a sample volume of 10 µL collected with a glass cuvette. Results are available in three minutes and the measuring range of HemoCue analyzer is 0.3-30.0 E9/L. Afinion AS100 CRP analyzer relies on immunometric membrane flow-through assay that measures CRP concentration quantitatively. The sample volume needed is 1.5 µL and results are delivered in 4 minutes. The measuring range of Afinion AS100 CRP is 5-160 mg/L in serum or plasma and 8-200 mg/L in whole blood. Venous blood samples for the reference methods were processed and analyzed in the hospital central laboratory.

In Study II, plasma PCT and CRP levels were determined in the hospital central laboratory as part of daily clinical routines using the Elecsys BRAHMS PCT assay (Roche Diagnostics, Mannheim, Germany) and the Modular P analyzer CRP assay (Roche Diagnostics, Mannheim, Germany).

In Study IV, white blood cell count and plasma CRP and PCT levels were determined immediately in the hospital central laboratory. Whole blood samples for MxA measurement were transported to the laboratory of clinical virology and stored in room temperature until lysed by hypotonic buffer 1:20. The samples were then stored in -70°C until the EIA analysis was performed as described earlier (Vallittu *et al.* 2008; Toivonen *et al.* 2015). Serum samples for TRAIL measurement were transported directly to the laboratory of clinical virology and stored in -70°C until the analysis was made by ELISA (Human TRAIL, Quantikine, R&D Systems Inc, Minneapolis, USA) according to manufacturer's instructions.

4.3 Virus diagnostic methods (III, IV)

Study III was a comparison between the multiplex antigen detection test and the reverse transcription PCR (RT-PCR) method. The mariPOC® antigen detection test system is a multiplex POCT providing qualitative results for eight respiratory viruses (influenza A virus, influenza B virus, RSV, adenovirus, human metapneumovirus (hMPV), parainfluenza type 1, 2, 3 viruses) from a single NPS sample. The test system is automated and allows random-access analysis. Antigen detection is based on two-photon fluorescence excitation technique accompanied by an immunometric assay (Koskinen *et al.* 2007). The preliminary results are available in 20 minutes and the final results in 120 minutes. A multiplex RT-PCR assay for respiratory viruses (Seeplex RV12; Seegene, Seoul, Korea) was used to detect influenza A virus, influenza B virus, RSV A and B, adenovirus, hMPV, parainfluenza type 1, 2, 3 viruses, rhinovirus A/B, and coronaviruses OC43/HKU1 and 229E/NL63. Random-hexamer primers (Fermentas, York, UK) were used in

synthesis of cDNA. Multiplex RT-PCR assay was used according to manufacturer's instructions. Sensitivity of the multiplex RT-PCR assay was low for influenza B in samples that were stored in -70°C and therefore an additional in-house PCR assay was used as a reference method for influenza B virus in frozen samples. The in-house PCR assay was performed as described earlier (Peltola *et al.* 2012). In addition, the samples that gave contradictory results by multiplex and in-house PCR assays for influenza B virus were tested by an in-house time-resolved fluoroimmunoassay (Nikkari *et al.* 1989). These results were in accordance with the in-house PCR results.

Respiratory virus diagnostics were performed by real-time RT-PCR from throat swabs and by serological methods in Study IV. A multiplex respiratory virus real-time RT-PCR test (Anyplex II RV16; Seegene, Seoul, Korea) was used for the detection of influenza A and B viruses, RSV A and B, adenovirus, hMPV, coronaviruses 229E, NL63 and OC43, parainfluenza type 1, 2, 3, 4 viruses, rhinoviruses, enteroviruses and bocavirus. In addition, an in-house real-time RT-PCR assay was used for the detection of rhinoviruses and enteroviruses (Osterback *et al.* 2013). Nucleic acid amplifications were performed by using Rotor-Gene 6000 or Qiagen Q (Qiagen, Hilden, Germany) instruments. Enteroviruses were genotyped with PCR from the viral protein 1 (VP1) region (Nix *et al.* 2006), and adenoviruses were genotyped from the hexon protein region (Ylihärsilä *et al.* 2013). Paired serum samples were stored in -70°C until analyzed. A commercial kit was used to detect EBV (Vidas, bioMerieux, France) IgG and IgM antibodies according to manufacturers' instructions. IgG antibodies against adenovirus, enteroviruses, influenza A and B viruses, parainfluenza virus types 1, 2 and 3 and RSV were analyzed by in-house enzyme immunoassays (Koskinen *et al.* 1987; Vuorinen and Meurman 1989). A μ-capture immunoassay was used to detect enterovirus IgM antibodies. For the detection of IgG and IgM antibodies, a mixture of coxsackievirus A16, coxsackievirus B3 and echovirus 11 antigens were used.

4.4 Bacterial diagnostic methods (IV)

In Study IV, 50 μL from the vortexed eSwab® transport media was used to perform bacterial culture on streptococcal-selective blood agar, standard blood, McLeod, Fastidious anaerobe agars and in a streptococcal-selective broth followed by subculture on streptococcal-selective blood agar. Bacterial colonies of interest were identified by standard methods, including MALDI-TOF (Bruker Daltonics, Bremen, Germany). ASO levels in serum were determined using the RapiTex® ASL kit (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany) according to the manufacturer's instructions. A commercial kit was used to detect *Mycoplasma pneumoniae* (Labsystems Diagnostics, Vantaa, Finland) IgG and IgM antibodies according to manufacturers' instructions.

4.5 Outcome measures and statistics

The main outcome measure in Study I was the agreement between POC and standard measurements of blood WBC and CRP levels. The Bland-Altman method was used to analyze this agreement (Bland and Altman 1986). In addition, intraclass correlation coefficients were determined.

Main outcome measures in Study II were the occurrence of discrepant plasma PCT and CRP results and the description of associated clinical conditions. The final classification of each case was based on the review of the patient records by the investigators. A Chi-square test, Fisher's exact test or Mann-Whitney U-test were used for statistical comparison of response variables between the two groups of cases with increased PCT and low CRP, and low PCT and increased CRP. These response variables were chosen because of their clinical relevance or because there are known differences in the kinetics between plasma PCT and CRP levels in these conditions. Our aim was to evaluate the significance of each variable by the multiple logistic regression model. However, that was not feasible because of the low number of cases of several variables in each of the groups.

The POC method for identification of multiple respiratory viruses was evaluated in comparison with RT-PCR by descriptive methods (study III). Sensitivities and specificities were calculated separately for each virus regardless of multiple virus detection. A *t*-test after Levene's test for equality of variances was used to compare the days from symptom onset to sampling between subgroups of children with true positive and false positive results. Diagnostic coverage of POC and RT-PCR methods were calculated separately for all viruses included in the multiplex PCR assay and for viruses that were included in the mariPOC® test system.

Main outcome measures in Study IV were the microbiological etiology of pharyngitis detected by bacterial culture, polymerase chain reaction or serology, and blood MxA level as a marker of host interferon response against virus infection. For the statistical comparison, all patients were classified into two etiological groups: patients with GAS or GAS-viral pharyngitis (GAS diagnosis based on bacterial culture) were classified as GAS and patients with all other etiologies or with an undetermined etiology were classified as non-GAS. Groups were compared by χ^2 or Mann-Whitney U tests. The area under the receiver operating characteristic (ROC) curve was determined for biomarkers to predict GAS or non-GAS etiology. Furthermore, patients were classified according to their microbial findings in six separate groups for descriptive analysis. Based on our earlier experience in young children, we used 175 µg/L as a cut-off level for increased blood MxA concentration (Toivonen *et al.* 2015).

All statistical analyses in all studies (I-IV) were performed using IBM SPSS Statistics Versions 21.0 or 22.0 (IBM Corp., Armonk, New York, USA).

4.6 Ethics

Study protocols of Studies I-IV were approved by the Institutional Review Board at the Clinical Research Center of the Turku University Hospital. Studies I and II were retrospective studies without any interventions and therefore no separate approval from the ethics committee or informed consent from patients was needed. Studies III and IV were prospective, observational studies. The Ethic Committee of the Hospital District of Southwest Finland approved the study protocol of Studies III and IV. Furthermore, informed consents were collected from the guardians of all patients in Studies III and IV.

5 RESULTS

5.1 Accuracy of WBC and CRP POC measurements (I)

5.1.1 Accuracy in the laboratory

In the laboratory validation study, the mean difference between POC and reference WBC count results was -0.4 E9/L (95% limits of agreement from -2.5 to 1.6 E9/L), meaning that the POC method gave, on average, a slightly higher result than the reference method. **Figure 1** presents these results graphically. However, a difference related to blood sampling method was noticed. The mean relative difference between POC and standard methods was larger in 24 micro-volume samples (-13%) than in 53 standard venous samples (-1%). Additional investigations showed that the two micro-volume samples that had the largest deviations (-82% and -93%) contained non-hemolyzed erythrocytes with Howell-Jolly bodies. Results given by the reference WBC count method that were included in this comparison ranged from 1.9 to 31.5 E9/L.

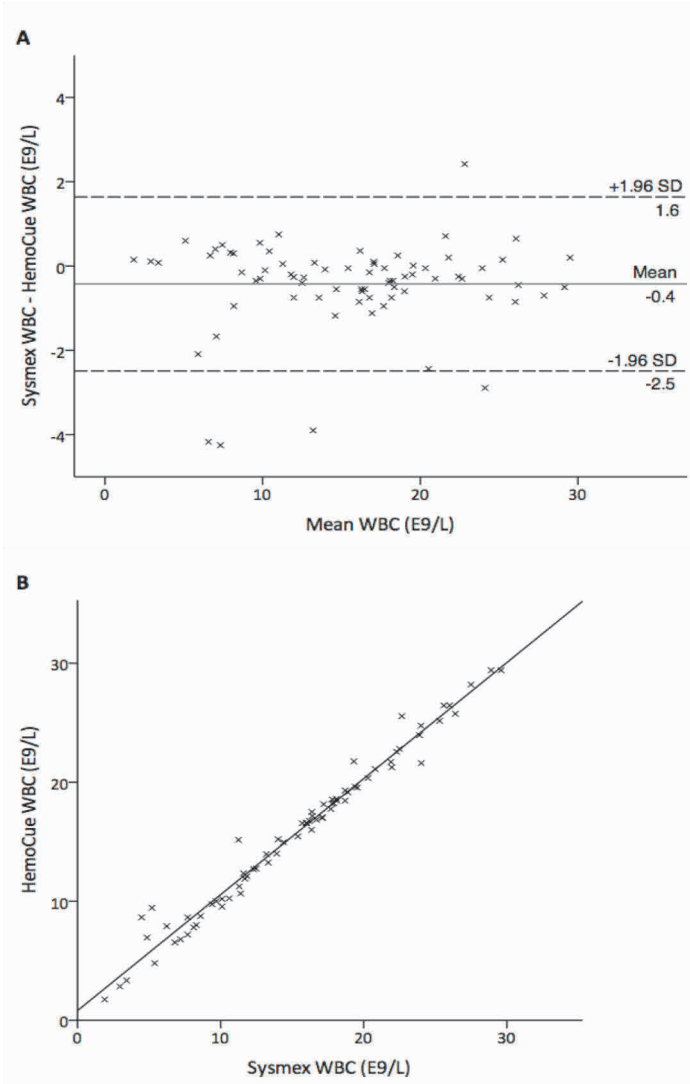

Figure 1. Bland-Altman plot showing the level of agreement (A) and scatterplot showing the linear correlation (B) for comparison of white blood cell count (WBC) measurements by HemoCue® point-of-care and Sysmex® tests in the central laboratory setting (n = 77). SD, standard deviation. Modified from the Study I.

The mean difference between POC and reference method CRP was 2.3 mg/L (95% limits of agreement from -5.5 to 10.1 mg/L) (**Figure 2**). The range of plasma CRP results measured by the reference method and included in this comparison was 5-159 mg/L. Intraclass correlations between POC and reference methods were 0.988 (95% CI 0.980-0.992) for WBC and 0.994 (95% CI 0.990-0.997) for CRP.

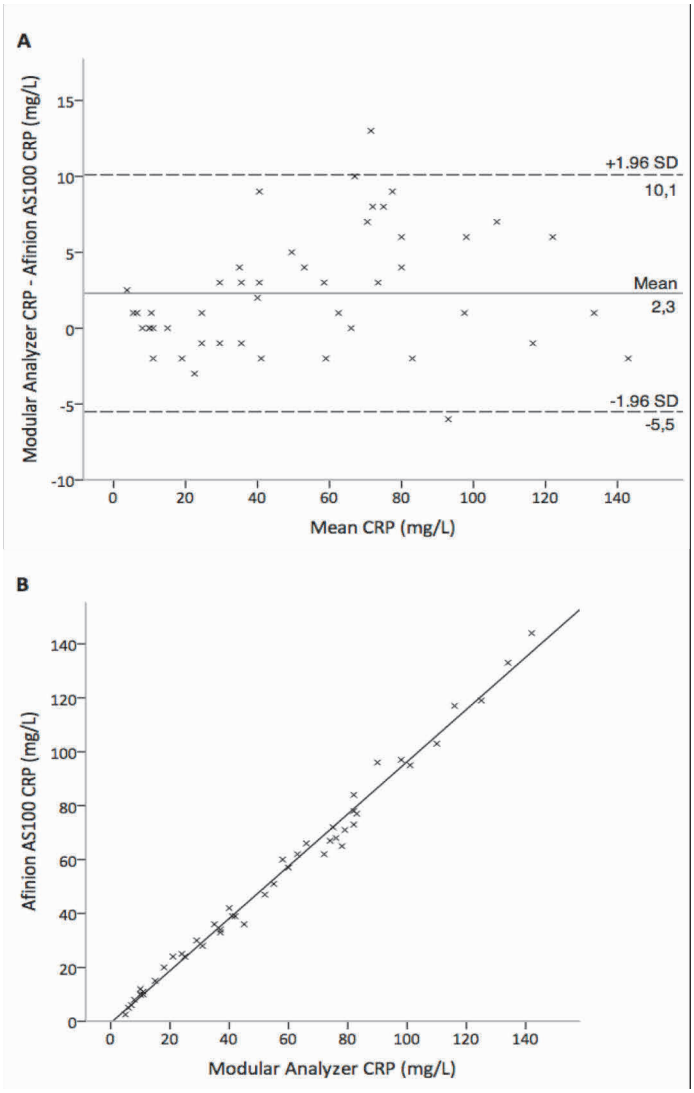

Figure 2. Bland-Altman plot showing the level of agreement (A) and scatterplot showing the linear correlation (B) for comparison of C-reactive protein (CRP) measurements by Afinion AS100® point-of-care and Modular® tests in the hospital central laboratory (n = 48). SD, standard deviation. Modified from the Study I.

5.1.2 Accuracy at the emergency department (ED)

In the clinical part of the study, eligible patients were retrospectively screened among 1989 documented POC measurements. Overall, 168 patients with 171 acute illness episodes were identified. The median age of patients was 2.4 years [interquartile range (IQR) 0.9-5.9 years], 54% were female and 87% were admitted to the hospital. Most of the patients had an acute infection.

The mean difference between POC and reference WBC count tests was 1.1 E9/L (95% limits of agreement from -6.5 to 8.8 E9/L) (**Figure 3**), and the mean difference between POC and reference CRP test results was -1.2 mg/L (95% limits of agreement from -29.6 to 27.2 mg/L) (**Figure 4**). The intraclass correlations between the two methods were 0.864 (95% CI 0.818-0.899) for WBC count and 0.967 (95% CI 0.955-0.976) for CRP.

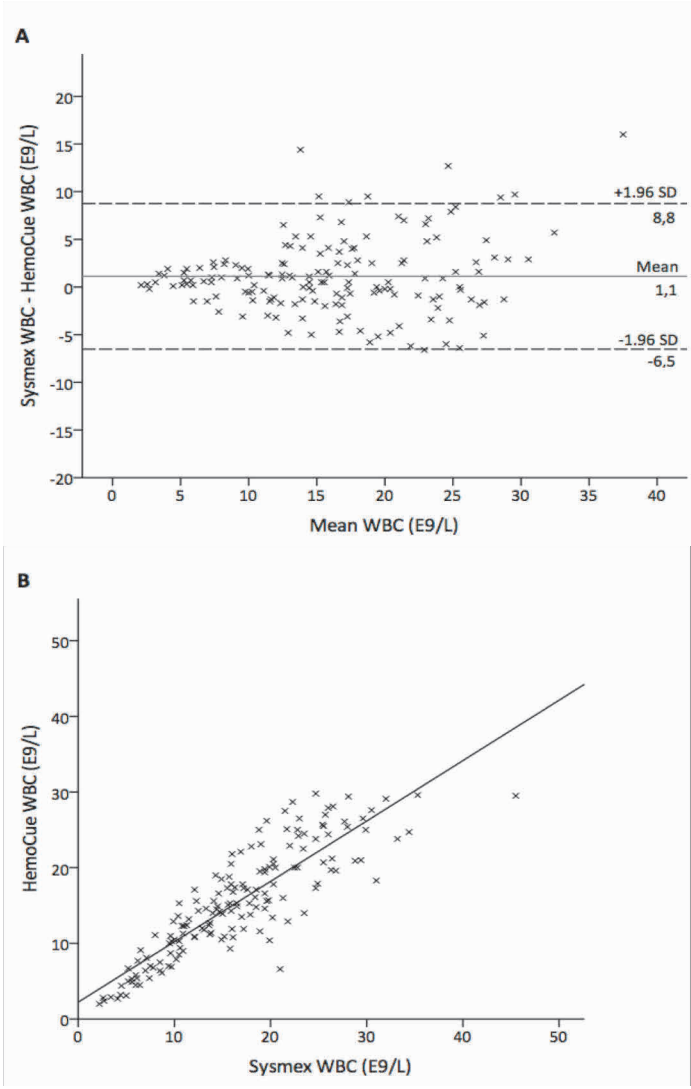

Figure 3. Bland-Altman plot showing the level of agreement (A) and scatterplot showing the linear correlation (B) for comparison of white blood cell count (WBC) measurements by HemoCue® point-of-care (POC) and Sysmex® tests in pediatric emergency department (ED) (n = 171 visits). Clinicians did POC testing at the ED in capillary blood samples. Sysmex WBC analysis was done from venous blood samples in the hospital central laboratory. SD, standard deviation. Modified from the Study I.

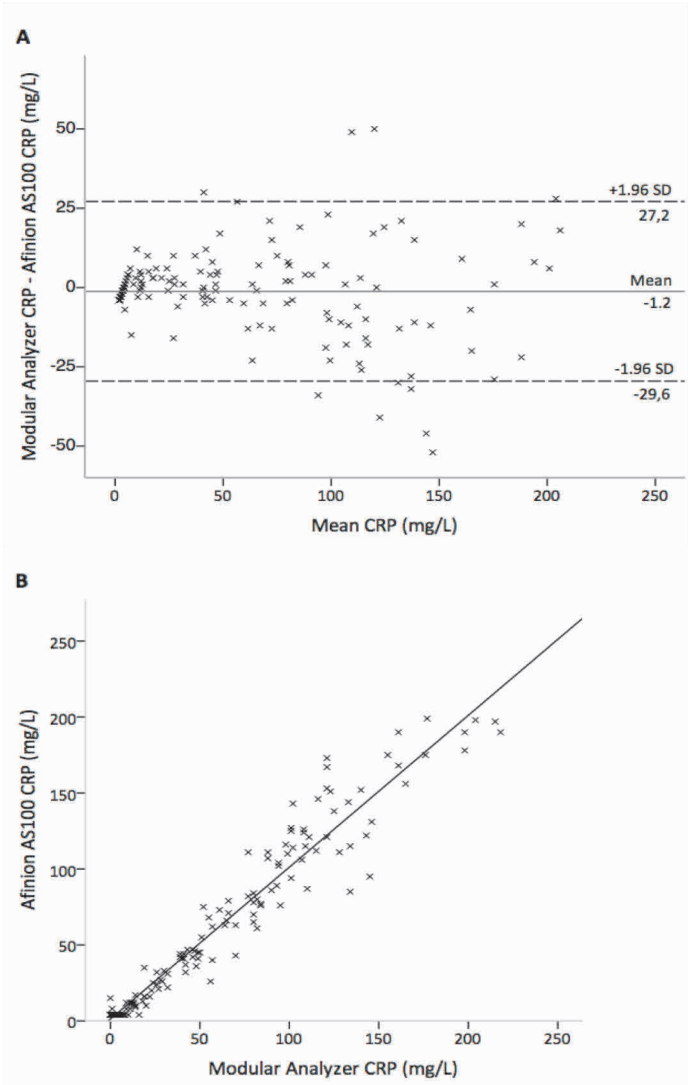

Figure 4. Bland-Altman plot showing the level of agreement (A) and scatterplot showing the linear correlation (B) for comparison of C-reactive protein (CRP) measurements by Afinion AS100® point-of-care (POC) and Modular® tests in pediatric emergency department (ED) (n = 171 visits). Clinicians did POC testing at the ED in capillary blood samples. Modular® CRP analysis was done from plasma separated from venous blood samples in the hospital central laboratory. SD, standard deviation. Modified from the Study I.

POC WBC results exceeded the analyzer's upper detection limit (>30 E9/L) in 14 patients. The respective WBC values measured by the reference method were 22.1-53.3 (mean 33.3 E9/L). Point-of-care CRP results exceeded the Afinion AS100® analyzers upper detection limit (>200 mg/L) in 24 patients. The respective CRP values measured by the reference method were 163-409 mg/L (mean 242 mg/L). Overall, POC CRP values were below the analyzer's measuring range (<8 mg/L)

in 34 patients. The respective CRP results measured by the reference method were 0-16 mg/L (mean 3.2 mg/L).

5.1.3 Clinical experiences

POC testing was relatively rapid to perform. Sampling required approximately 1 minute of hands-on time. Additional 3 and 4 minutes were needed for processing by HemoCue@ WBC and Afinion AS100® CRP analyzers, respectively. The test results were available within 5 minutes and technical errors were rare. We did not measure the ED patient flow in this study, but our experience was that POC testing made the patient care proceed more fluently.

5.2 Discrepancy of plasma PCT and CRP levels (II)

A review of the hospital laboratory records showed a total of 1240 simultaneously analyzed plasma PCT and CRP samples during the study period. After excluding the serial measurements, 635 cases in 536 patients were documented. The median age of the children was 3.6 years and 45% of them were female.

5.2.1 Occurrence

The occurrence of discrepant plasma PCT and CRP levels was 29%. Only the plasma PCT level was elevated in 75 (12%) cases, and only the plasma CRP level was elevated in 107 (17%) cases. Both biomarkers were elevated in 149 (23%) cases, while the concentration of both PCT and CRP remained low in 304 (48%) cases.

5.2.2 Associated clinical conditions

The group of patients with high PCT and low CRP included more patients with fever for less than 24 hours (37 vs. 12), neonates (17 vs. 0), bacteremic patients (8 vs. 0) and patients with hypoxia or HD stress (11 vs. 0) than did the group of patients with low PCT and high CRP. The latter group had a greater number of patients with isolated bacterial infections (3 vs. 18), non-infectious inflammatory illnesses (1 vs. 12) and postoperative setting (1 vs. 19) (**Table 4**). Patients with high PCT and low CRP were younger (median age 1.3 years) in comparison with patients who had low PCT and high CRP (median age 7.7 years).

Table 4. The number (%) of patients with selected clinical characteristics/findings according to the PCT (high ≥ 0.5 μg/L) and CRP (high ≥ 40 mg/L) concentrations in plasma. Modified from the Study II.

Characteristic	*Study population (n=635)*	*PCT high, CRP low (n=75)*	*PCT low, CRP high (n=107)*	*p Value**
Febrile < 24 h	151 (100)	37 (25)	12 (8)	<0.001
Neonate 0-6 days	27 (100)	17 (63)	0 (0)	<0.001
Bacteremia	27 (100)	8 (30)	0 (0)	0.001
Hypoxia or HD stress	27 (100)	11 (41)	0 (0)	<0.001
Isolated infection	46 (100)	3 (7)	18 (39)	0.009
Inflammatory	19 (100)	1 (5)	12 (63)	0.016
Postoperative	60 (100)	1 (2)	19 (32)	<0.001
Pneumonia	109 (100)	16 (15)	25 (23)	0.747
Pyelonephritis	44 (100)	3 (7)	8 (18)	0.529

PCT, procalcitonin; CRP, C-reactive protein; HD, hemodynamic.
*Univariate comparison between the two groups with high PCT, low CRP and low PCT, high CRP.

In total, 27 bacteremic patients were identified. Gram-positive bacteria caused 17 (63%) cases and gram-negative bacteria 10 (37%) cases. The two most common pathogens were *Streptococcus pneumoniae* (n = 4) and *E. coli* (n = 4). Fifteen patients with bacteremia had high PCT and CRP levels, while PCT alone was elevated in eight patients and in four patients none of the biomarkers increased. The duration of symptoms was < 24 hours in six of the eight bacteremic patients with high PCT and low CRP. Conversely, there were 37 patients with high PCT and low CRP whose symptoms appeared less than 24 hours before sampling. Of these, six (16%) had bacteremia. Most of the patients who had a confirmed virus infection had low plasma PCT and CRP levels. Adenoviral infections were often (7/15 cases, 47%) associated with elevated CRP but low PCT levels. Acute diabetic ketoacidosis (DKA) was associated with elevated CRP levels in two of four and with elevated PCT levels in three of four cases. None of the patients had any clinical sign of bacterial infection.

5.3 Multianalyte antigen detection POC test for respiratory viruses (III)

In total, samples from 158 children were tested by both mariPOC® POCT in the ED and by PCR in the laboratory of clinical virology. The median age of the patients was 1.8 years (IQR 0.6-7.1 years), 43% were female and little less than half (44%) were admitted to hospital. The majority of the patients (76%) had no underlying medical conditions and 76% were febrile (body temperature ≥38°C).

5.3.1 Diagnostic performance

The mariPOC® antigen detection test system was positive for at least one of the test's eight viruses in 69 samples (44%). In comparison, the RT-PCR method was positive for at least one of these eight viruses in 84 (53%) and at least for one of the twelve viruses, which are included in the multiplex RT-PCR assay, in 104 (66%) samples (**Figure 5**).

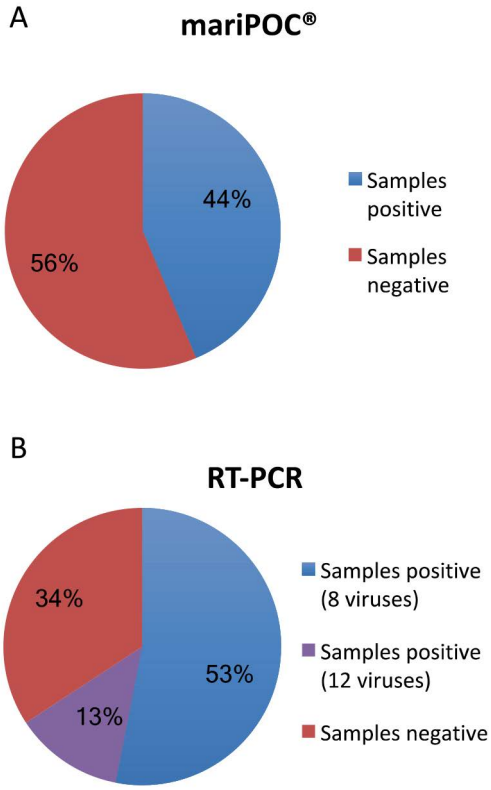

Figure 5. Diagnostic yield of mariPOC® (A) and RT-PCR (B) in 158 nasopharyngeal swab samples. Portion of RT-PCR positive samples are shown for both 8 viruses (viruses covered by mariPOC®) and 12 viruses (all viruses in the multiplex RT-PCR assay). RT-PCR, reverse transcription polymerase chain reaction.

The sensitivity and specificity of the mariPOC® test in comparison with RT-PCR was calculated separately for each virus (**Table 5**). Because of the low number of patients with parainfluenza virus detection the analytical performance of mariPOC® test system could not be assessed reliably for these viruses.

Table 5. Sensitivities and specificities according to virus of mariPOC® in comparison with RT-PCR in 158 children. Modified from the Study III.

	MariPOC®	*RT-PCR*		*% (95% confidence interval)*	
		Positive	***Negative***	***Sensitivity***	***Specificity***
Influenza A virus	Positive	5	0	71 (38-100)	100
	Negative	2	151		
Influenza B virus	Positive	19	3	86 (72-100)	98 (95-100)
	Negative	3	133		
Adenovirus	Positive	3	4	25 (1-50)	97 (95-99)
	Negative	9	142		
Respiratory syncytial virus	Positive	31	0	89 (78-99)	100
	Negative	4	123		
Human metapneumovirus	Positive	4	0	50 (15-85)	100
	Negative	4	150		
Parainfluenza virus 1	Positive	0	0	N/A	N/A
	Negative	0	158		
Parainfluenza virus 2	Positive	0	1	N/A	N/A
	Negative	2	155		
Parainfluenza virus 3	Positive	1	0	N/A	N/A
	Negative	2	155		

RT-PCR, reverse transcription polymerase chain reaction; N/A, not applicable.

5.3.2 Feasibility in the ED

The positive mariPOC® results were available in the preliminary phase (20 minutes from the beginning of the analyses) in 76% of the cases in which at least one virus was positive in the final results. In 1% of the cases, at least one virus was reported preliminary positive, but the final result was reported negative. mariPOC® test system was regarded easy to use by the ED staff. The final results were not reported by the test system in four patients (3%).

5.4 Etiology of febrile pharyngitis (IV)

The study on the etiology of febrile pharyngitis (IV) included 83 children and adolescents. The median age of the study subjects was 5.5 years (IQR 3.2-12.2 years), 46 (55%) of them were male and all were treated as outpatients. Two-thirds of the patients (69%) returned for a follow-up visit 10-40 (median 18 days, IQR 17-21 days) days after the enrollment.

5.4.1 Clinical presentation

According to the case criteria, all patients were febrile and had exudates (57%) and/or intensive redness (74%) in their oropharynx. Overall, 73% of patients had

been febrile for three days or less. Cervical lymphadenopathy was reported in 51 (61%) patients, sore throat in 70 patients (95% of patients who were capable of reporting any symptoms, n = 74) and cough or rhinitis in 50 (60%) patients. A McIsaac score could be determined in 66 patients with sore throat and all of them had scores between 2 and 5 (median 4, IQR 3-4).

5.4.2 Microbiological etiology

A microbial cause of pharyngitis was documented in 73 (88%) children. GAS was detected in the throat swab samples of eight (10%) patients and together with viruses in 11 (13%) patients. Group C streptococcus (GCS) or group G streptococcus (GGS) was detected alone in two (2%) and together with viruses in three (4%) patients. One or more viruses, without group A, C or G β-hemolytic streptococci, were detected in 49 (59%) patients. The etiology of the pharyngitis remained unknown in 10 (12%) patients. The etiological results are presented in **Figure 6**.

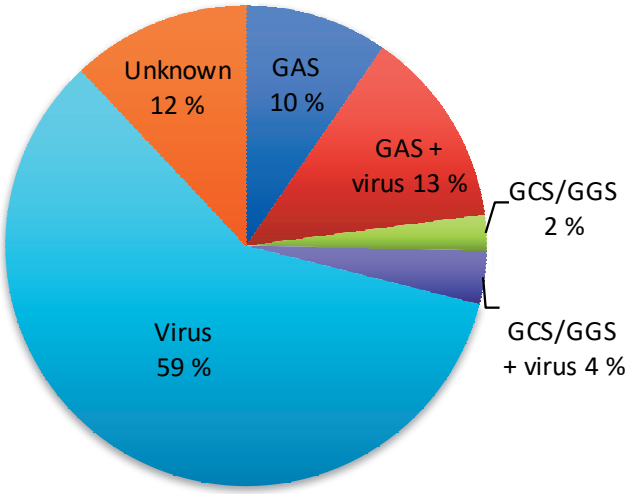

Figure 6. Microbiological etiology of febrile pharyngitis (n = 83). GAS, group A streptococcus; GCS, group C streptococcus; GGS, group G streptococcus.

The diagnosis of β-hemolytic streptococci (GAS, GCS, GGS) was made based on standard culture on a streptococcal-selective blood agar plate except in one patient in whom it was diagnosed by enrichment culture only. The diagnosis of virus infection was made by RT-PCR alone in 44 patients, by RT-PCR together with antibody response in paired serum samples in fifteen patients and by serology alone in four patients. Multiple respiratory viruses were detected in 20 patients.

Enteroviruses were detected in 21 children (25%) and were the most frequently detected viral pathogens followed by rhinovirus (in 18 (22%) children) and adenovirus (in 13 (16%) children). Most of these virus findings were together with other viruses: 13/21 for enterovirus, 13/18 for rhinovirus and 10/13 for adenovirus. All four coronavirus OC43 findings were together with other viruses. By using serology results together with cycle threshold data from PCR analyses, we were able to estimate the most important virus finding in 12/20 (60%) of the patients with multiple virus detection.

Mycoplasma pneumoniae IgM antibodies together with IgG antibodies could be detected in the initial serum samples of ten patients. Seven of them were from 14-16 years old patients. Paired serum samples were available from four of these patients and showed no IgG antibody response between the samples. GAS or virus was detected along with *M. pneumoniae* IgM antibodies in 9/10 of the patients.

5.4.3 Biomarkers

Levels of five different biomarkers were measured in blood, plasma or serum in all pharyngitis patients: WBC, CRP, PCT, MxA and TRAIL. PCT level was the only biomarker that showed no statistically significant difference between patients with and without GAS detection (**Table 6**). Blood MxA levels were increased in most (79%) of the patients with virus detection but not in patients with a sole GAS/GCS/GGS finding (10%). Blood MxA concentrations were elevated also in most of the patients with unknown microbial etiology.

Table 6. Comparison of mean biomarker levels [95% confidence interval] in group A streptococcus (GAS) and non-GAS pharyngitis (n = 83). In addition, blood biomarker levels for patients with a sole virus finding are shown. Modified from the Study IV.

Biomarker	*GAS (n = 19)*	*Non-GAS (n = 64)*	*P**	*Sole virus (n = 49)*
WBC (E9/L)	13.3 [11.2-15.4]	10.2 [8.8-11.6]	0.005	9.9 [8.3-11.5]
CRP (mg/L)	38 [21-54]	22 [14-29]	0.01	19 [11-27]
PCT (µg/L)	0.22 [0.11-0.33]	0.33 [0.22-0.45]	0.65	0.31 [0.19-0.41]
MxA (µg/L)	298 [164-432]	996 [660-1332]	<0.001	953 [739-1167]
TRAIL (pg/mL)	98 [77-119]	149 [124-175]	0.04	161 [131-192]

GAS, group A streptococcus; WBC, white blood cell count; CRP, C-reactive protein; PCT, procalcitonin; MxA, myxovirus resistance protein A; TRAIL, tumor necrosis factor related apoptosis-inducing ligand.

*Univariate analysis by the Mann-Whitney U test between GAS and non-GAS pharyngitis

6 DISCUSSION

6.1 Infection biomarkers

Biomarkers for early identification of sepsis in febrile patients have been sought vigorously for decades. A need for a timely diagnosis was fortified by data demonstrating the direct proportionality of early antibiotic initiation and survival in sepsis (Nobre *et al.* 2007). To meet this challenge, evidently accurate and feasible rapid tests for clinically relevant biomarkers are needed. In children, the high prevalence of viral infections means that the most important clinical question in febrile patients in the ED is: viral or bacterial? Therefore, biomarkers that could help specifically address this question are in great demand.

6.1.1 POC tests for WBC and CRP (I)

WBC and CRP are unquestionably among the most widely used biomarkers in diagnostics of infectious diseases. This is true despite the obvious limitations that both of these biomarkers have concerning their ability to ascertain serious infections (Van den Bruel *et al.* 2011). Rapid CRP testing has the potential in aiding patient flow, cost-effectiveness and in decreasing unnecessary antibiotic prescriptions (Cohen *et al.* 2006; Aabenhus *et al.* 2014; Kokko *et al.* 2014; Nijman *et al.* 2015). To my knowledge, only few pragmatic studies on the accuracy and feasibility of CRP and WBC POC testing at the site of care have been performed. These earlier studies do not report the differences in the accuracy of the tests between laboratory-based and bedside-based evaluations (Esposito *et al.* 2005; Cohen *et al.* 2006; Monteny *et al.* 2006; Casey and Pichichero 2008; Verbakel *et al.* 2014).

In our study, we first performed the methodological evaluation and validation in the hospital central laboratory. In this first part of the study, we found that use of WBC POC measurement in neonates, especially in those born preterm, might be compromised by the presence of Howell-Joly bodies in the peripheral blood. This finding is hardly relevant for the POC test use in the ED setting but must be considered regarding neonates and especially preterm infants. However, the main finding was that both WBC and CRP POC tests gave good analytical accuracy in these conditions.

After implementing the tests in routine use in the pediatric ED, we analyzed data from patients who had WBC and CRP results available by POC and standard laboratory methods. In this clinical part of the study, POC results differed more from the results given by the standard methods than in the first part of the study. In the first part, methodological comparison was conducted in a more controlled

laboratory environment explaining these differences. Still, accuracy of both POC tests was clinically satisfactory also in the ED setting.

Our results highlight the importance of the pragmatic performance of the studies, validation at the site of use and thorough quality control concerning POCTs (Drancourt *et al.* 2016). Furthermore, we found that WBC and CRP POCTs are feasible, their analytical accuracy is acceptable for ED use and that the results were consistently available in five minutes enabling more effective clinical decision making to take place and thus improving patient flow.

6.1.2 PCT and CRP measurements (II)

Data on PCT has expanded tremendously during the past 5-10 years. It has proven to be a useful biomarker in detecting patients with serious bacterial infection and to control antibiotic treatment in intensive care units. Kinetics of biomarker blood levels favor PCT over CRP because of the more rapid increase after onset of infection and decrease following recovery. The more rapid elevation of plasma PCT levels compared with CRP was demonstrated also in our study in patients with blood culture positive infections. PCT, unlike CRP, was elevated in 71% of the bacteremic patients who were presented to the hospital in less than 24 hours from the symptom onset. It can be speculated that prospective studies focusing on this time frame could find PCT as an even more valuable biomarker in comparison with CRP.

The nonspecific elevation of plasma CRP level in illnesses not related to bacterial infection is well-established. Virus infections, especially adenovirus infections, can increase plasma CRP levels and recent reports suggest that also influenza A H7N9 virus can make CRP levels elevate (Putto, Meurman, *et al.* 1986; Wu *et al.* 2016). We now confirmed our earlier findings that CRP more often than PCT was elevated in infections caused by adenovirus (Elenius *et al.* 2012).

Observations or studies concerning the nonspecific elevation of plasma PCT levels in pediatric patients are far more scarce than that of CRP with the exception of physiologically elevated plasma PCT levels in newborns (Lapillonne *et al.* 1998). In adults, PCT has been reported to elevate in the absence of bacterial infection following major stress such as trauma, surgery or cardiac arrest (Reinhart *et al.* 2012). In our study, we observed that three out of four children presented to the hospital with an acute DKA had elevated plasma PCT levels without other signs of bacterial infection. It can be speculated that the major HD stress that often accompanies acute DKA and/or elevated blood levels of TNF or other cytokines result in increased concentrations of PCT in plasma. Also CRP was elevated in two of the DKA patients, a phenomenon already well-described (Dalton *et al.* 2003; Karavanaki *et al.* 2011).

In this study, we addressed the question about the occurrence and clinical relevance of discrepant plasma CRP and PCT results in hospitalized children. We found out that discrepant results are not uncommon and that the physician who uses these biomarkers must be familiar with the pitfalls related to them.

6.1.3 MxA and TRAIL (IV)

MxA is a promising biomarker of virus infection. Earlier studies used a laborious detection method from peripheral blood lymphocytes necessitating cell separation (Halminen *et al.* 1997; Koskenvuo *et al.* 2006). More recent studies used an EIA method enabling a potential for point-of-care test development. A French study compared blood MxA levels in clinically distinct patient populations (Engelmann *et al.* 2015). In this study, researchers showed that MxA is elevated in virus infection in contrast to serious bacterial infection. So far, TRAIL has been studied as a biomarker of virus infection only in combination with other biomarkers (Oved *et al.* 2015; Eden *et al.* 2016; van der Does *et al.* 2016).

In our study on febrile pharyngitis (IV), no symptoms, signs or clinical decision rules could reliably differentiate GAS from non-GAS etiology. This phenotypical homogeneity is one of the strengths in our study population to evaluate the validity of biomarkers for differentiating viral and bacterial etiology. An earlier study by our group showed that blood MxA level increases in symptomatic but not in asymptomatic virus infections (Toivonen *et al.* 2015). This finding is in accordance with another study concerning transcriptional differences in subjects with symptomatic and asymptomatic rhinovirus infection (Heinonen *et al.* 2016). In our present study, we could demonstrate that febrile pharyngeal infections that were caused solely by group A, C or G betahemolytic streptococci were not associated with a marked elevation of blood MxA concentration. This was not the case in patients in whom both streptococci and viruses were detected. If these GAS-viral co-detections occurred because of an actual virus infection with coincidental detection of pharyngeal GAS carriage, because of an actual GAS infection with coincidental virus detection or because of a real concomitant viral-bacterial (super)infection remains to be solved. However, an increase in blood MxA levels in most of these patients indicates an active interferon and hence antiviral response.

We did the statistical comparisons concerning various biomarkers and clinical characteristics between groups with a GAS finding (GAS group) and without a GAS finding (non-GAS group) conservatively regardless of possible GAS-virus co-detection in the GAS group. We did this because of the above-mentioned uncertainty of the clinical relevance of GAS detection in these patients and because earlier studies have not specifically divided patients into those with GAS or those

with both GAS and virus detected. If we would have made the comparisons with GAS only and virus only groups, the performance of MxA would have been remarkably better. In addition to MxA, increased serum TRAIL concentrations were associated with virus detection. Nevertheless, MxA outperformed TRAIL in differentiating between GAS and non-GAS etiology by ROC analysis in our material.

In our study, we used blood MxA level as an indicator of active type I or III interferon response presumably triggered by an acute virus infection. We used it to evaluate the relevance of virus findings. In addition, blood MxA level showed good potential as a virus infection specific biomarker. Future studies should focus first on extending the knowledge of blood MxA levels in other clinical scenarios than pharyngitis and to confirm the test's specificity for viral infection only. The final aim should be in demonstrating the potential impact of MxA testing on clinically meaningful outcomes such as antibiotic use or hospital admission rates.

6.2 Microbiological diagnostics

6.2.1 Multianalyte antigen detection POC test for respiratory viruses (III)

Virus diagnostics has changed irrevocably during past decades. Virus culture, which used to be the gold standard for virus diagnosis, is now only infrequently used in routine clinical diagnostics. First, various tests based on antigen detection and more recently on NAAT have become the standard for diagnosing most of the virus infections. In 2011 when our study on RADT for multiple respiratory viruses was introduced, no multiplex NAAT tests for point-of-care use were available. This is in contrast to how it is now. However, multiplexed assays seem a rationale way to address the needs of respiratory virus diagnostics especially during the peak winter season because epidemics of different viruses typically overlap with each other. Virus diagnostics in children with respiratory tract infection could reduce the use of antibiotics and additional investigations, diagnose RSV to better predict the clinical course of illness, provide antiviral treatment of influenza and better cohort the admitted patients.

Our results show that the test system (mariPOC®) was feasible for use in pediatric ED and that it performed moderately well in detecting influenza viruses and RSV. Three-fourths of all virus-positive results were available in 20 minutes and the rest in 2 hours. Sensitivity for influenza A or B during an epidemic was 83%, which is not sufficient for patients with severe influenza-like illnesses. A negative influenza RADT result must be confirmed by PCR in these patients and empirical anti-viral treatment is recommended before the influenza PCR results are available (CDC 2016). An advantage of the multiplex RADT in comparison with traditional

influenza RADTs is the possibility to find other viruses such as RSV at the same time. The accuracy of clinical influenza diagnosis in children, and especially in infants, is low even during the epidemic (Peltola *et al.* 2005). Already now, most pediatricians feel that microbiologic confirmation of RSV infection has clinical relevance. It can be predicted that when the new RSV antivirals reach the clinic, the importance of rapid and accurate diagnosis will be increasingly emphasized (Mejias 2015).

Despite the increasing use of NAATs in the diagnostics of virus infections, antigen detection still has some advantages on its side. First, most of the antigen detection tests are simple, cheap and therefore widely available. Lateral flow assays suit POC use by non-laboratory-trained staff well. This was true also with the more automated test system that we had in our study, because clinical personnel regarded the test easy to use. Second, respiratory viruses can be found by NAATs also from asymptomatic individuals which complicates the interpretation of positive results. This is true especially for adenovirus. A recent study suggested that high viral burden (low cycle threshold in RT-PCR) indicates true symptomatic adenovirus infection, especially in the absence of co-detection of other viruses (Song *et al.* 2016). In our study material, we had only 12 adenovirus positive cases by RT-PCR. In these patients, the sensitivity of antigen detection test for adenovirus was 25% in comparison with the multiplex RT-PCR assay. Furthermore, most of our patients who were positive for adenovirus by RT-PCR, but negative by antigen detection, had multiple viruses detected. If what we found in our study by antigen detection is similar to what Song et al. found in their study, showing that, in some patients, the RT-PCR test was clinically over-sensitive for adenovirus, can only be hypothesized. However, the low number of adenovirus cases in our study makes it impossible to draw firm conclusions on this subject.

The multianalyte antigen detection test system was specific, relatively sensitive for detection of influenza and RSV, and easy to use. If there will be need for this, or any other tests based on respiratory virus antigen detection when the novel, multiplexed POC NAATs become more widely available, remains to be seen.

6.2.2 GAS throat culture and serology (IV)

Throat culture on a BAP is currently the gold standard for diagnosis of GAS pharyngitis. RADTs can be used, but some guidelines recommend a backup culture for negative results (Shulman *et al.* 2012). In our study on febrile pharyngitis, we did the standard culture on a streptococcal-selective BAP. In addition, the sample was inoculated in a streptococcal-selective broth followed by the standard culture to enhance the sensitivity. However, only in one of the nineteen patients with GAS isolates, the diagnosis was made based on growth in the streptococcal-selective

broth only. In all other patients, the standard throat culture yielded a positive result. Our data suggests that additional microbiological culture procedures are not routinely needed concerning the diagnosis of GAS pharyngitis.

Experts' opinions are divided concerning a need for backup throat culture after negative RADT. So far, no studies have assessed the potential harms or benefits of not performing a confirmatory throat culture in a randomized controlled trial. Another issue concerning some of the NAATs and novel antigen detection tests is the increased rate of GAS detection in comparison with throat culture (Cohen *et al.* 2013, 2015; Vakkila *et al.* 2015; Pritt *et al.* 2016). We do not present any data regarding antigen detection tests for GAS here. However, based on the existing literature, it seems reasonable to assume that a rapid, easy-to-use and at most moderately more sensitive method in comparison with throat culture would be ideal for the routine diagnosis of GAS pharyngitis. Preferably the test should also be cost beneficial.

Serology is not suitable for routine diagnostics of GAS pharyngitis. It is not clear how well serology works for diagnosis of GAS pharyngitis in research use either. An Australian family study defined >0.2 log-10 rise of ASO or ADB titer between acute and convalescent serum sample as an unequivocal serological confirmation of GAS pharyngitis. In the same study, GAS pharyngitis was likely serologically confirmed in patients with serum ASO or ADB levels greater than normal in samples taken >7 days after the infection onset (Danchin *et al.* 2007). We had only five patients with an ASO titer increase between the paired serum samples of whom three were GAS culture positive, one was GCS culture positive and one was culture negative for streptococci. Considering the classical description that suggests that ASO titers peak in 3-5 weeks, it is possible that the sampling interval between paired serum samples might not have been sufficiently long enough for an optimal detection of ASO titer rise in our study (Steer *et al.* 2015). Determination of ADB levels in addition to ASO might have also improved the performance of serological detection of streptococcal pharyngitis (Johnson *et al.* 2010). However, current serological methods for identification of acute GAS infection are far from ideal.

6.3 Etiology of pharyngitis

Clinical decisions and guidelines concerning diagnostics, treatment or prevention are based on the knowledge of causes of the diseases, their etiology. In infectious diseases, these causative agents are microbes, either viruses, bacteria, fungi or parasites. The microbiological etiology of pharyngitis has been studied widely. Earlier studies found out that number of different viruses, streptococci and occasionally also other bacteria were detected in oropharynx of patients with

symptomatic pharyngitis (Evans and Dick 1964; Glezen *et al.* 1967; Moffet *et al.* 1968; Kaplan *et al.* 1971). Later, etiological studies in children used mainly culture, serological and antigen detection methods for identification of potential pathogens (Putto 1987; Esposito *et al.* 2004). More recent studies have focused on diagnostics and treatment of GAS (Krober *et al.* 1985; Danchin *et al.* 2007; Little *et al.* 2013). Current scientific interest concerning pharyngitis focuses mostly on overuse of antibiotics (Barnett and Linder 2014; Dooling *et al.* 2014; Fleming-Dutra *et al.* 2016).

In our study, we aimed to complete the picture of the microbiological etiology of pharyngitis by using NAATs in detecting viruses and by evaluating the clinical relevance of virus findings by measuring the level of antiviral biomarkers from blood. We did not seek all potential pathogens by quantitative or single-analyte NAATs, methods that potentially would have been more sensitive than multiplexed or serological assays. Still, we did not look either for CMV or *Fusobacterium necrophorum* by PCR. Otherwise, the diagnostic coverage of potential pathogens was comprehensive in our study. We confirmed the earlier observations that most of the pharyngitis cases with non-GAS etiology are caused by respiratory viruses. Modern diagnostic assays allowed swift identification of these viruses in the vast majority of cases.

Our data fortifies the important role of viruses in the etiology of pharyngitis in especially the youngest children. However, 18% of children less than 5 years old had GAS detected in their throat, though most of these children had viruses detected as well. Pharyngitis caused by GAS is not common in this age group, but it certainly is possible. Another slightly unexpected finding was that enteroviruses were the most often detected viral pathogen. Enteroviruses are a known cause of hand-foot-and-mouth disease and also a potential pharyngitis pathogen (Shulman *et al.* 2012). There was an enterovirus epidemic in Finland in August-November 2014 that partly explains these findings (Jaakola *et al.* 2015). We used an in-house RT-PCR assay (Osterback *et al.* 2013) together with serology for the detection of enteroviruses which might have led to greater diagnostic sensitivity in comparison with earlier etiological studies. Interestingly, there was a global enterovirus D68 outbreak in 2014 causing morbidity mainly due to lower respiratory infections in Turku, Finland as well. However, none of the enteroviruses that were genotyped in our study were type D68 suggesting a minor role that they play in pharyngitis.

Comprehensive etiological studies on acute illnesses that are mainly treated in outpatient clinics are done infrequently, probably due to the difficulties in patient recruitment. In this respect, our study produced highly valuable data on the general view of the etiology of pharyngitis, on the co-detection of GAS and viruses and on the potential of a simple MxA blood test in demonstrating the significance of virus detection.

6.4 Limitations

There are some important limitations in this thesis. First, we did not have a separate study clinic for any parts of the work, but data was collected during normal patient care by varying hospital personnel. It can be argued that our approach might endanger the implementation of convergent inclusion criteria and further complicate the interpretation of the results. However, our pragmatic study design probably represents better the real-life environment and thus might ease the clinical implementation of the results. Second, all studies were at a single center and small scale which justifiably raises questions concerning the generalizability of the results and emphasizes the need for larger, multicenter trials. Finally, patients for Studies I and II were identified retrospectively based on available laboratory results and, thus, the clinical picture of these patients was heterogeneous. For Study II, the lack of blinding of the investigators for biomarker results was an additional drawback. On the other hand, our approach allowed us to make unexpected observations, such as the marked increase of plasma PCT levels in patients with acute DKA.

6.5 Future of diagnostics in pediatric infectious diseases

Recently, Cioffredi and Jhaveri summarized the current standing and future prospects concerning diagnostics in febrile children (Cioffredi and Jhaveri 2016). Increasing use of multiplex NAATs at the POC and the role of transcriptomic methods seem possible and even probable diagnostic approaches. What the authors do not refer to is the use of biomarkers as diagnostic tools. My personal suggestion is that gene expression screening accompanied by proteomics could be used to identify the best diagnostic biomarkers. The added advantage of using proteins as biomarkers instead of mRNA transcripts is the potential for development of a simple, robust and inexpensive POCT. However, systematic clinical evaluation of a child with an assumed acute infection remains the backbone of all decisions concerning patient care. Also clinical judgement rules, like the Rochester criteria, are continuously relevant to the exclusion of SBIs, and especially beneficial in combination with rapid virus testing (Byington *et al.* 2004, 2012).

Need for POCTs in infectious diseases is highlighted by the emergence of AMR (Marston *et al.* 2016). Antimicrobial stewardship, one of the key elements in combating the AMR, utilizes several different POCTs. This is to first confirm that the indication for antibiotic use is appropriate and later, identify the pathogen allowing clinicians either to stop, narrow, continue or change the course of antimicrobial treatment. Determining every means that we can use to spare patients from unnecessary use of antibiotics is of greatest importance.

Diagnostics of GAS pharyngitis is in transition. If novel GAS POCTs that are more sensitive than traditional throat culture can identify new groups of patients who are likely to benefit from antimicrobial treatment remains to be seen. The only way to solidly demonstrate the potential effect would be a randomized, placebo-controlled treatment trial. Because the benefits of antibiotics for pharyngitis with current, less sensitive diagnostic methods are relatively minor, it can be speculated that such trials should include thousands of patients to be statistically powerful enough to demonstrate the efficacy of the drug in an extended group of patients. Currently, a study of that size does not seem feasible. Before clinical benefits of wider antibiotic use for pharyngitis are studied, it is unjustified to broaden the treatment criteria.

As mentioned before, NAATs are increasingly “the diagnostic tests of choice” for a variety of clinical syndromes. It seems probable that the future of respiratory virus diagnostics will rely mainly on multiplex NAATs performed at the site of care completed by a high quality virological laboratory facilities for more advanced and confirmatory diagnostic functions. Consequently, the use of antigen detection techniques might decrease in well-developed healthcare systems. However, the price that we pay for enhanced sensitivity of NAATs is the complicated interpretation of positive results, especially when multiple viruses are detected (Nascimento-Carvalho and Ruuskanen 2015). Furthermore, it is tempting to speculate if antigen detection tests, which detect only high virus loads, could more efficiently than NAATs identify the true causative virus.

7 CONCLUSIONS

The objectives of this thesis were to assess the accuracy and clinical feasibility of POC WBC and CRP measurements at the pediatric ED (I), to determine the occurrence and clinical significance of discrepant plasma PCT and CRP levels in acutely ill children (II), to evaluate the diagnostic performance and practicality of a POC multianalyte respiratory virus antigen detection test performed at the pediatric ED (III) and to study the microbiological etiology of febrile pharyngitis in children and the meaning of virus detection by simultaneous measurement of blood MxA level (IV). An ultimate goal is to improve diagnostic procedures in children with acute infections enabling better targeted antimicrobial treatment.

In Study I, we demonstrated the importance of evaluating the performance of POCTs pragmatically at the actual site of care. We showed that the accuracies of POC WBC and CRP tests are satisfactory for their function as the frontline laboratory tools. The relevance of our findings is emphasized by the widespread use of these tests.

The occurrence of discrepant plasma PCT and CRP levels in acutely ill children was found to be common (Study II). In addition to confirming earlier observations of more rapid rise of PCT than CRP in bacteremic patients, we showed, for the first time, that acute DKA is associated with an elevation of plasma PCT levels in the absence of bacterial infection.

In Study III, we found that a multianalyte antigen detection POCT for respiratory viruses was suitable for ED use. It performed moderately well for detection of influenza viruses and RSV, but it was not sensitive for detecting adenovirus in comparison with PCR.

In Study IV, we documented viruses as the most common cause of febrile pharyngitis in children and adolescents. In many other recent studies on pharyngitis, GAS has been the only microbial pathogen sought. Because diagnostic investigations in our study covered also viruses, the etiological picture as a whole, but also the role of GAS, is now explained in more detail. Our results reinforce the current practice of sparing patients with non-GAS illness from antibiotic treatment. In addition, blood MxA has the potential to become a biomarker of acute virus infection.

ACKNOWLEDGEMENTS

This work was done at the Department of Paediatrics and Adolescent Medicine at the University of Turku and Turku University Hospital during 2008-2016.

I thank Professor Erika Isolauri, MD, as the Head of the Department for giving me an opportunity to work as a PhD student at the Department of Paediatrics, at University of Turku. The Department of Emergency Services in Turku University Hospital is acknowledged for allowing me to do the pharyngitis study there.

I would like to thank warmly my supervisor Professor Ville Peltola, MD, for giving me a great role model as a physician scientist in the field of paediatric infectious diseases (PID). He is a skillful clinician and a highly professional researcher who has always had time for me and my work. In addition, he has allowed me space for professional growth during my project.

I am grateful for the reviewers of this thesis, Docent Tarja Heiskanen-Kosma, MD, and Docent Janne Aittoniemi, MD, for their time and expertise regarding the thesis manuscript. They are among the few people who have actually read the book. I thank Robert Badeau, PhD, for the effective language checking of my thesis. I'm thankful also for Dean Pentti Huovinen, MD, from my PhD advisory committee.

I thank all my co-authors in Turku University Hospital and in University of Turku for their contribution to this work: Pia Leino, MD and Professor Ilkka Mononen, MD, at Tykslab; Docent Tytti Vuorinen, MD, Docent Matti Waris, PhD, and Riikka Österback, PhD, at the Department of Clinical Virology; Docent Kaisu Rantakokko-Jalava, MD, at the Department of Clinical Microbiology; Docent Jukka Hytönen, MD, at the Department of Medical Microbiology and Immunology. Special thanks to my collaborators at the Department Paediatrics. Jussi Niemelä, MD, has been an important coworker and a mate during the whole PhD project. I was privileged to work together with one of the big names in the international PID community, Professor Terho Heikkinen, MD. Varpu Elenius, MD, an excellent pediatrician, teamed up with me in the procalcitonin project. Johanna Lempainen, MD, has brought a great deal of laboratory expertise into our research team with her. I thank research nurse Ulla Torkko for her help and company in the pharyngitis study. I thank all the children and their families who participated in the study, as well. I think that kids, in general, are the most wonderful and amusing people.

I show my appreciation to my boss, the Head of the Department of Paediatrics and Adolescent Medicine in Turku University Hospital and my co-author, Professor Jussi Mertsola, MD. I admire his farsightedness and thank him sincerely for helping me to set out my professional direction. I thank Professor Emeritus Olli

Ruuskanen, MD, and Turku University Hospital Research Foundation for offering me a place to work at the Foundation's Research Unit. Professor Ruuskanen has founded the field of PID in Finland together with other ambitious and stubborn individuals. For me, he has been a collaborator, a mentor and a friend.

I express my gratitude to all my fantastic work colleagues at the Department of Paediatrics and Adolescent Medicine in Turku University Hospital. I respect all the nurses and doctors in our hospital for their positive approach to my project and to science overall. I'm grateful also to the terrific people in the Department of Pediatrics in Central Finland Central Hospital. I recognize also my fellow-Tutkari-people for their company during the years.

We are a nice team of young clinicians/scientist in the group of Professor Ville Peltola. I wish to credit Laura Toivonen, MD, Sinikka Karppinen, MD, Tamara Teros-Jaakkola, MD, Linnea Schuez-Havupalo, MD, Maria Hartiala, MD and more recently Mohamed Yahya, MD, and Ville Forsström, medical student, for being a part of our small community. Special thanks for my friend Santtu Heinonen, MD, for being my closest colleague, even though we have never worked together. On top, cheers to friends at PIBID/TIDIRP/R2D2.

I thank all my friends for being a friend to me. This includes, but is not limited to, people from Turku 2008 pop-rock scene; Turun Partio-Sissit ry; Alvar Saints; The Pilgrimage; C. Antabus and the metropolises of Jyväskylä, Parainen and Piippola.

I am grateful for having a family like I do. I have a very helpful and easygoing family-in-law, a smart and fun sister-in-law, a brother that is my best friend, parents that have a view of life that I admire them for, sons that are the two most important things for me in the world, and a wife whom I respect, enjoy spending time with and am in love with.

This work was financially supported by Turku University Foundation, Research Funds from Specified Government Transfers, Research Grant of Turku University, The Finnish Medical Foundation, University of Turku Graduate School, The Sohlberg Foundation, The Pediatric Research Foundation, The Orion Corporation Research Foundation and The Turku University Hospital Research Foundation.

Turku, January 2017

Lauri Ivaska

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