The puzzle of pediatric infections

Challenges and opportunities for improving diagnosis of children with suspected infection

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THE PUZZLE OF PEDIATRIC INFECTIONS

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The batik motif shown in this thesis is called lung-lungan combined with the wings of the Garuda. Lung-lungan, from the Javanese tulung tulungan meaning 'to help', contains elements of leaves and flowers connected by their tendrils and symbolizes growth, supportiveness, and togetherness. The Garuda, a mythical bird originally found in Hindu mythology, symbolizes determination and knowledge.

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The Puzzle of Pediatric Infections

Challenges and Opportunities for Improving Diagnosis of Children with Suspected Infection

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CONTENTS

FOR IBRAHIM ISA, MURTI NIGRUM SUWARDI
& KLAAS KEUNING

Chapter I

General introduction and thesis outline

SOLVING THE PUZZLE OF PEDIATRIC INFECTIONS

Suspicion of infection is one of the most frequent presentations in a pediatric emergency department.[1] In general, antimicrobial treatments and immunization programs during childhood have improved the burden of pediatric infection to a great extent. Although many advancements have been made in the field of pediatric infection, such as the development of bacterial biomarkers and rapid viral testing, early recognition and diagnosis still remains a challenge. The puzzle of pediatric infection is distinguishing severe infections, with potential life-threatening consequences, from the more prevalent self-limiting and harmless infections. When a child is presented in the emergency department with fever and no source is identified after a full history and physical examination, this is called fever without a source (FWS). About 6 to 15% of these cases is caused by a severe viral or bacterial infection which can lead to long-term sequelae or even mortality in case of delayed treatment.[2, 3] The other 85 to 94% is caused by mild viral infections which occur frequently in children. The latter cause is self-limiting and a vital part of immune system development whereas the first requires immediate antimicrobial treatment to improve patient outcomes.

To recognize and diagnose these severe infections, – or to 'solve the puzzle' – a physician collects all relevant pieces of the puzzle (figure 1). Relevant information is acquired through evaluation of risk factors for disease and other vulnerabilities of the patient, patient history, physical examination, and the disease course.[4] This is put in the context of background knowledge of the epidemiology of pathogens, the differential diagnoses and clinical practice guidelines to decide on the appropriate diagnostic testing. Diagnostic testing for FWS mostly involves sample collection to identify the causing pathogen and radiologic imaging can support localization of the infection focus. However, challenges in recognition, in sample collection and in the use of FWS guidelines can hamper the physician in solving the puzzle of forming the diagnosis. Exploring these challenges from the perspective of specific pediatric infections in addition to the perspective of children with FWS may reveal opportunities to improve current management of suspected pediatric infection.

CHALLENGES OF RECOGNITION

Adequately evaluating the patient's vulnerability and history, physical examination and the disease course are essential for early recognition of (severe) infections. Age, comorbidity and other host risk factors for infectious disease informs the physician on the patient's vulnerability to severe infections. Particularly in young children additional relevant information can be acquired from perinatal information and maternal antenatal screening.

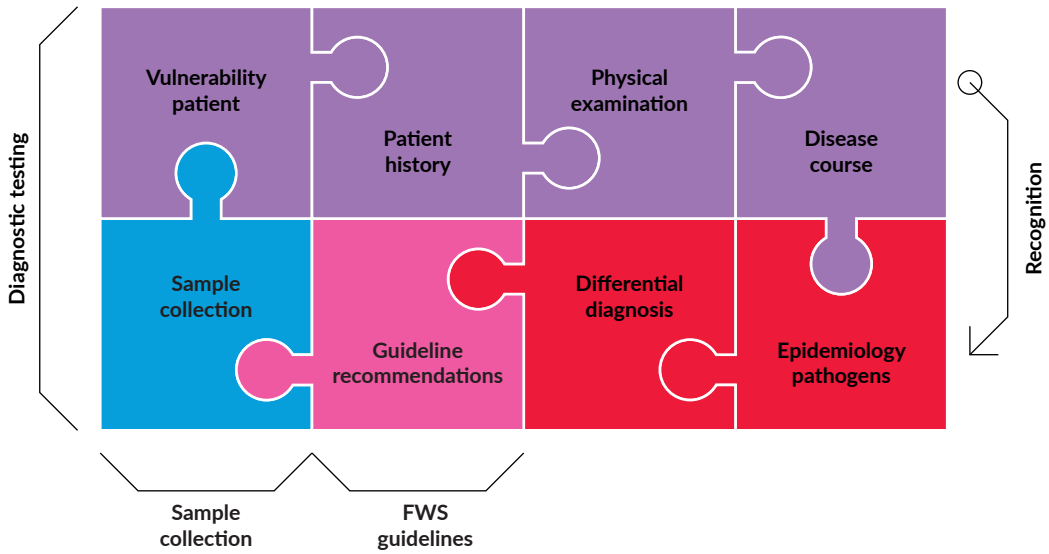


Figure 1 The puzzle of pediatric infections

Many research efforts have been made in search of the most accurate predictors of severe infection.[5, 6] Complicating these efforts, the patient history and physical examination, or clinical presentation of pediatric infection is often characterized by fever without any localizing or characteristic features, as is the case in FWS. Fever can also be absent at the initial presentation in the emergency department in both severe and self-limiting infections.[7, 8] Moreover, the clinical presentation of several rare severe infections is challenging for physicians. Particularly for less experienced physicians recognition of rare infections or distinction between ill- and well-appearing patients can prove to be challenging.[9-11] Even when a characteristic feature is present, this can be difficult to recognize when it involves a rare infectious disease that a physician is not often familiarized with, such as syphilis. A skin rash in a febrile child, for example, could be a diagnostic clue in a small subset of severe infectious diseases.[12] Since a skin rash also often occurs in mild viral infections, specific knowledge of morphology is needed to recognize that rash as a diagnostic clue. This non-specific presentation impairs early recognition and diagnosis of well-known infections and new emerging infections alike. Evaluating multiple aspects of hampered recognition in infectious diseases can identify opportunities for improvement of prompt diagnosis and treatment.[4]

CHALLENGES OF SAMPLE COLLECTION

In order to solve the puzzle of promptly diagnosing severe infections, the physician should be aware of the epidemiology of pathogens, the differential diagnosis and relevant guideline recommendations to choose the appropriate diagnostic microbiological testing. Microbiological testing requires the collection of samples from bodily fluids or tissue to detect the source of a pediatric infection. In response to the invasion of a pathogen in a certain part of the body, the host reacts with both a local and systemic immune response that can also be detected in several sample types. The correct sample type to diagnose the source of infection is dependent on the pathogen and the phase of the infection, and may require samples of multiple bodily fluids or tissue. Importantly, the physician should weigh the benefit and harm of diagnostic testing for the patient. Often young children do not only present with non-specific features, they are also more vulnerable to severe infection such as a urinary tract infection, bacteremia or meningitis compared to older children.[13] This combination leads to an almost five-fold increased use of diagnostic and therapeutic resources and health care costs in children younger than three months compared to children older than six months.[9]

Despite the development of many guidelines and risk prediction tools, variation in practice is still substantial in the management of children with FWS.[14] Although some variation in practice can be explained by differences between patients or populations, unwanted practice variation also occurs due to differences in diagnostic practice between physicians leading to unnecessary testing.[15] Differences in physician's preferences but also knowledge and experience impact variation in FWS management: less experienced physicians for example showed a 3.2-fold higher emergency department resource use than their experienced colleagues.[9] Detecting variation in sample collection from different perspectives can pinpoint further opportunities for improving diagnosis.[16] Challenges of sample collection can thus be explored by describing variation in practice in confirmed severe pediatric infections, particularly in case of rare infections. In addition, this can be approached by evaluating variation in sample collection and diagnostic testing from the perspective of children presenting with FWS.

Certain sampling methods can be painful and invasive investigations such as venous punctures are considered a burden by patients and their parents.[17] Parents have varying attitudes towards risk when managing FWS, either going to great lengths in order to avoid the discomfort of sampling or accepting no risk at all of missing severe infections.[18] Underutilization of relevant diagnostic testing is, however, an underappreciated yet common issue in the diagnostic process as well.[19] In order to inform the patient on the indication and diagnostic value of certain sampling methods the physician should consider the differential diagnosis of potential infections. By evaluating the probability of the considered diagnoses and verifying whether the clinical presentation is consistent with these diagnoses and their epidemiology, the physician can minimize overuse of diagnostic testing and, thus, the burden on patients.[4] Another way forward in improving diagnostic testing for pediatric infections is exploring less-invasive sampling methods. Development of non-invasive sampling methods is of value to alleviate the burden of diagnostic testing in children with pediatric infection.

CHALLENGES OF FWS GUIDELINES

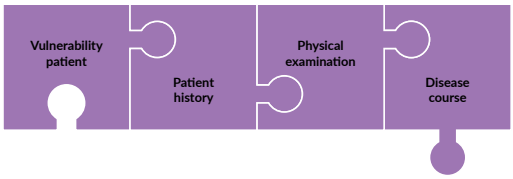
To comprehend the vast expending evidence on all pieces of the puzzle and balance the benefits and impact of diagnostic testing, several countries have developed national FWS guidelines. These clinical practice guidelines aim to systematically collect relevant evidence to guide physicians in performing the appropriate diagnostic workup. The purpose of clinical practice guidelines in general is to enable consistent and cost-effective evidence-based health care. Several national pediatric guidelines are examples of improved health care in terms of patient outcomes, resource use or cost-effectiveness.[20, 21] However, without evaluating guidelines in current practice, it remains unknown whether this purpose is fulfilled. In a study of hospital guidelines in the United States, the availability of a guideline was not associated with reduced costs and in some cases even associated with increased costs.[22] An illustrative example is the Dutch guideline on pediatric minor traumatic head injury, providing recommendations for cerebral imaging. While a decrease of unnecessary scans was intended and expected after implementation of the guideline, a guideline evaluation study reported the opposite: an increase in the number of performed scans without improvement in clinical outcome.[23] Evaluating current clinical practice provides insight in challenges of FWS guidelines and assists in the improvement of clinical practice guidelines for early recognition and diagnosis of severe infection.

Objectives

A. What are challenges of recognizing severe pediatric infections?
B. What are challenges of sample collection in suspected pediatric infection?
C. What are challenges of clinical practice guidelines for suspected pediatric infection?

To comprehend these pieces of the puzzle of recognition and diagnosis in suspected pediatric infection, several objectives were formulated. The first part of this thesis addressed challenges of recognition and sample collection in several infectious diseases (objectives A and B). From the extremely rare and severe to the common and mild, from the old to the new infections, several aspects of hampered recognition and diagnosis in congenital syphilis, neonatal herpes simplex virus (HSV) infection and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) were described. The second part of this thesis focused on challenges of clinical practice guidelines (objective C), assessing current practice in children presenting with FWS in emergency departments in the Netherlands and comparing FWS guidelines of high-income countries.

RECOGNITION AND DIAGNOSIS OF CONGENITAL SYPHILIS



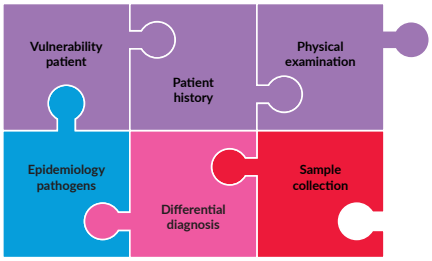
An illustrative example of a severe infection posing challenges for recognition and diagnosis is congenital syphilis, also known as 'the great imitator'. Congenital syphilis is caused by the spirochete bacterium *Treponema pallidum* and can be vertically transmitted at any time during pregnancy. Despite being a very well-treatable disease, congenital syphilis still occurs and is even on the rise in high-income countries such as the United States, Canada and New Zealand.[24-26] Its nickname refers to the many clinical presentations of congenital syphilis which among others can affect the skin, eyes, liver, kidneys, bones, hematopoiesis and nervous system. This spectrum of disease manifestations hampers early recognition, since even the most distinctive feature of rash has a diverse morphology, with half of patients showing no rash at all.[27] Moreover, as a consequence of the non-specific presentation and the rare incidence, awareness among physicians regarding the clinical presentation and diagnostic resources is low.[11] To increase awareness among physicians in high-income countries, **chapter 2** presents a case of early congenital

syphilis with a very severe and almost fatal disease course and provides a review of diagnosis and treatment. In response to objective A, **chapter 2** demonstrates that congenital syphilis was not recognized by many specialized physicians despite an elaborate patient history, physical examination and sample collection for diagnostic testing. As mentioned in the review, the most important intervention in preventing congenital syphilis is adequate antenatal syphilis screening. **Chapter 2** shows that syphilis can still be transmitted between screening and childbirth in a mother without any risk factors for sexually transmitted infections. Congenital syphilis should be considered in the differential diagnosis of any severe and challenging infectious disease case presenting with skin lesions, even in the context of a negative antenatal syphilis screening.

Addressing objective B, this case report also illustrated several challenges in sample collection for congenital syphilis. Spreading and replication of syphilis spirochetes can cause inflammatory responses in the skin and throughout the body. To diagnose congenital syphilis the sample collection is dependent on the phase of the infection. In early primary infection *Treponema pallidum* can be identified directly through darkfield microscopy, PCR testing or direct fluorescent antibody staining of skin or bodily fluid samples.[28] As lesions may be absent or self-limiting, serological serum testing remains the most important step of diagnosing an active infection. An important challenge demonstrated by **chapter 2**, is that even if skin lesion samples are collected, the diagnosis can be missed if syphilis is not considered in the differential diagnosis and no specific staining is performed.

RECOGNITION AND DIAGNOSIS OF NEONATAL HERPES

The incidence of the severe neonatal HSV infection – although fortunately still low – is showing a slightly increasing trend in the Netherlands from 2.4 per 100,000 newborns in the nineties to 4.8 in the latest measurements of 2012 to 2015.[29] Neonatal HSV infection is caused by HSV type 1 or 2, which are enveloped double stranded DNA viruses and part of the Herpesviridae family. Although most primary HSV infections occur asymptomatically in childhood, a primary infection in neonates can lead to localized skin, eye, mouth disease, encephalitis or disseminated disease.[30] HSV can be transmitted from mother to child peri-partum through maternal viral shedding or less commonly intrauterine or post-partum.



Maternal viral shedding in the genital tract often occurs asymptomatically, complicating early recognition of neonatal HSV infection.[30] Further complicating recognition: the initial presentation of this uncommon yet severe neonatal infection can vary, with a proportion presenting without any classical symptoms such as skin lesions, seizures or critical illness.[31] An important consequence of delayed recognition is delayed treatment, which has been shown to significantly increase mortality of neonatal HSV infection.[32] Incidence rates have regularly been described in the Netherlands, however the clinical presentation and outcomes remained unknown.[29] In addition to the variation in clinical presentation, neonatal HSV infection is characterized by the importance and timing of sample collection when an infection is suspected. Diagnostic testing in suspected neonatal HSV can range from PCR, viral culture, immunofluorescence and serology which can be performed on samples of skin lesions, mucosal swabs, cerebral

spinal fluid or blood. Early in the infection PCR testing of cerebral spinal fluid samples can still be negative for HSV. Importantly, there is no national consensus on which sample types should be collected in which patients, which can lead to substantial practice variation. A previous study in the Netherlands has shown that diagnostic management in neonates with a suspicion of HSV infection was often inadequate and inconsistent.[33] However, due to the rarity of this infection, none of the children in this study were diagnosed with an HSV infection. For objective A and B , **chapter 3** therefore evaluates recognition challenges and the variation in sample collection in a retrospective case series of confirmed neonatal HSV patients in two medical centers in the Netherlands.

RECOGNITION AND DIAGNOSIS OF SARS-COV-2

The new SARS-CoV-2 emerged in 2019, causing a pandemic with a profound impact on society. This single-stranded RNA virus often causes mild or asymptomatic infection in children and can also present as FWS.[34] Occasionally, SARS-CoV-2 can cause a more severe coronavirus disease 2019 (COVID-19) or multi-inflammatory syndrome in children (MIS-C) requiring hospital or even intensive care unit admission. Hospital admission, however, is rarer in children compared to adults. An important challenge is recognizing the pediatric COVID-19 patient who is vulnerable for a more complicated disease course requiring hospital admission. Pre-existing comorbidity was identified as an important predictor for severe disease and hospitalization in adult patients.[35] This association of comorbidity and disease severity has similarly been suggested in children, although evidence supporting this notion was often based on admission rates only instead of a detailed evaluation of the clinical disease course.[36] Children with comorbidity may be admitted earlier than healthy children as a precautionary measure, which is likely to bias the measured associations. Thus, for objective A **chapter 4** evaluates the association between comorbidity and disease severity in pediatric SARS-CoV-2 infection.

Despite the generally mild clinical presentation, children have a contributory role in transmission of SARS-CoV-2 within a population. With many asymptomatic infections among children, symptom-driven PCR testing of nasopharyngeal samples lead to an underestimation of the epidemiology in children. It is important to evaluate epidemiology to estimate the burden of new emerging infectious diseases and to establish effective public health measures for children. Humoral immunity, which can be studied to estimate the epidemiology, is generally measured in serum. However, this method of sampling requires venous punctures and thus poses a burden on children. Exploring less-invasive sampling methods can decrease the burden of testing and increase willingness of the population to participate in antibody surveys. To address this challenge of sample collection, **chapters 5 and 6** explore the value of saliva sampling as compared to serum sampling for detecting humoral immunity against SARS-CoV-2 in children.

NATIONAL GUIDELINES FOR RECOGNITION AND DIAGNOSIS

As the previous chapters demonstrated, pediatric infections show a great variation in clinical presentation, etiology and diagnostic testing. Current literature informs on many pieces of the puzzle of recognition and diagnosis which may be relevant in the management of FWS. The Dutch Association of Pediatrics published a national FWS guideline in 2013, based on relevant literature and on the recommendations of the National Institute for Health

and Care Excellence (NICE) guideline from the United Kingdom.[37] The Dutch guideline provides a practical step-by-step pathway to differentiate between a self-limiting infection and a severe infection requiring treatment. The aim is to improve early recognition of severe pediatric infections without overuse of diagnostic resources. In the ongoing process of health care improvement, systematical evaluation and validation are crucial elements in the health care quality circle.[38] As most guidelines are based on studies performed in other countries or different populations, the applicability in current practice and the clinical outcomes remain unknown without evaluation and external validation.[4] Thorough evaluation of adherence to the Dutch national guidelines "Fever in secondary care setting in children aged 0-16 years" and its clinical outcomes has not yet been performed. Therefore, **chapters 7 and 8** aim to improve the management of children with FWS through a retrospective and a prospective multicenter evaluation of the national guideline in current practice. Addressing objective C, these chapters measured adherence to the diagnostic and therapeutic recommendations of the guideline in Dutch hospitals. In addition, the adherence and non-adherence group were compared to detect practical bottlenecks and patterns in non-adherence required for future targeted guideline improvement.

Several studies have shown significant variation in FWS management in high-income countries despite the availability of national guidelines.[14, 39] A multicenter study reported wide variation between European emergency departments in prescriptions of broad-spectrum antibiotics in febrile children.[40] Non-adherence to guidelines and variation in practice can be explained by several factors, including inconsistency in definitions and recommendations between existing guidelines.[4, 41] To explore this challenge of inconsistencies between FWS guidelines for objective C, in **chapter 9** the definitions and the diagnostic and therapeutic recommendations published in national and regional FWS guidelines of high-income countries are compared.

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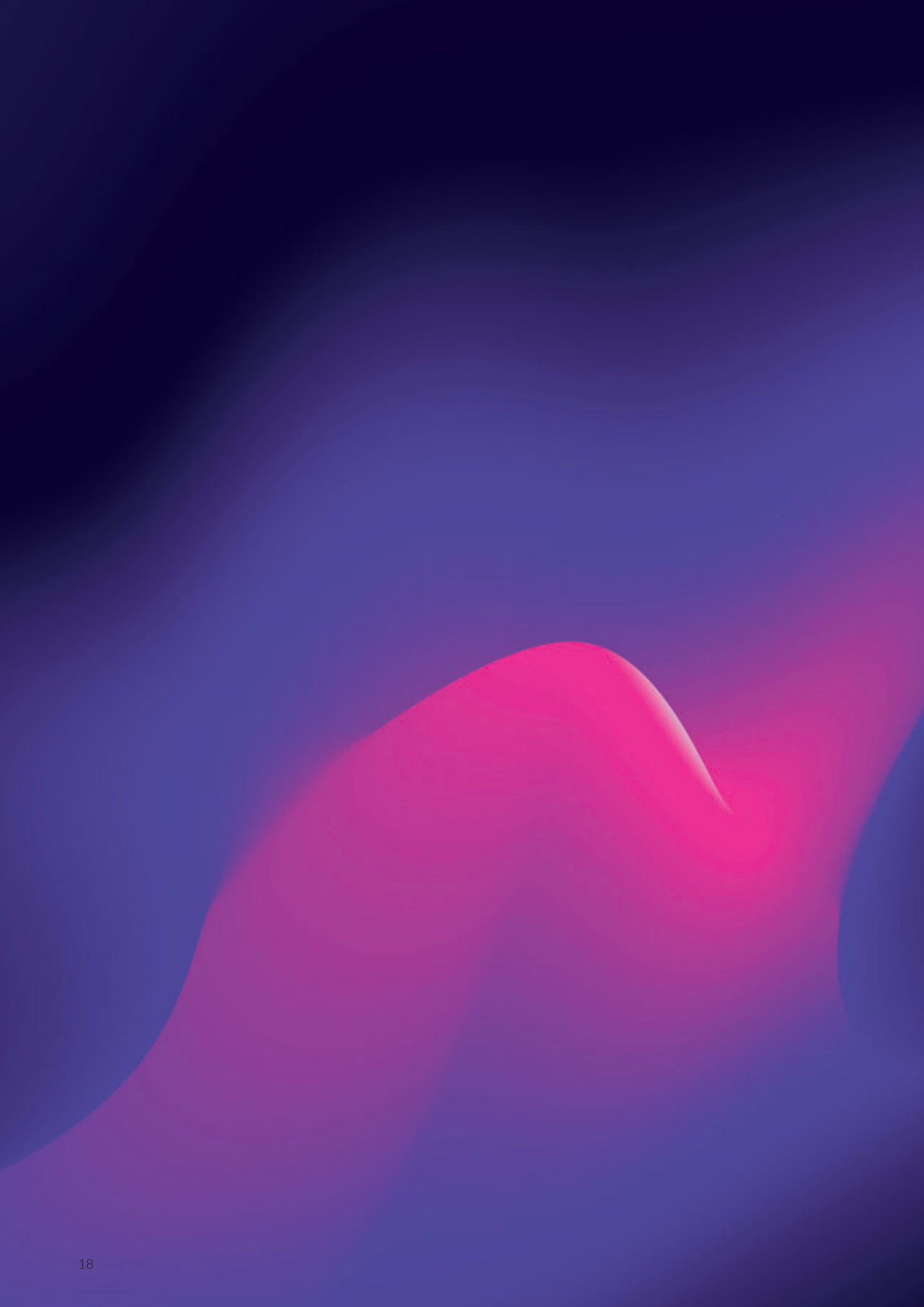

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Challenges and opportunities in pediatric infections

Chapter II

Congenital syphilis, the great imitator – a case report and review

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ABSTRACT

Syphilis is caused by a spirochete bacterium called ***Treponema pallidum***. Vertical transmission of spirochetes can lead to congenital infection of the fetus in pregnant women who are inadequately treated or not treated at all, causing a variety of clinical manifestations including stillbirth, neonatal death, or asymptomatic infection as well as the more commonly recognized manifestations.

We present a severe case of a 3-month-old boy with skin lesions, portal hypertension and anemia. As the mother was tested negative for syphilis antibodies at 16 weeks of gestation, a diagnosis of congenital syphilis was initially not considered. This case demonstrates that transmission of ***T. pallidum*** can still occur in developed countries with a high coverage rate of antenatal screening. Early recognition may be hampered if physicians do not consider congenital syphilis altogether.

Congenital syphilis should be considered in any severe and diagnostically challenging infectious disease case even in the context of negative antenatal screening.

INTRODUCTION

Acquired syphilis is known as a sexually transmitted infection, caused by a spirochete bacterium called *Treponema pallidum, subspecies pallidum. T. pallidum* can cause a chronic infection with separate clinical stages, following transmission through sexual contact (including oral contact) or through transfusion of unscreened blood or blood products (for a review see ref 1). Mother-to-child transmission of spirochetes can lead to congenital infection at any time during pregnancy if an acquired syphilis infection is not identified and adequately treated with penicillin. Congenital syphilis can present as asymptomatic infection as well as a variety of clinical manifestations, from stillbirth or intra-uterine growth restriction to early and late symptomatic disease in infants.[2] The only treatment for neonates or infants with confirmed or suspected infection is penicillin, aiming to cure disease and thus prevent sequelae of congenital infection.[3]

Incidence of congenital syphilis infection appears to be decreasing in several countries with the introduction of preventive antenatal screening by the World Health Organization (WHO).[4, 5] Although syphilis is a preventable and treatable disease, a steady increase in the rate of congenital syphilis has been described even in developed countries, such as the United States.[6] Fluctuation in incidence of congenital syphilis is known to be associated with the incidence of syphilis in women of reproductive age.[6] In the Netherlands, coverage of antenatal screening for syphilis is high, and the case rate of congenital syphilis very low.[7] This low incidence of congenital syphilis in the Netherlands, combined with the wide range of clinical signs and symptoms of disease, may hamper prompt recognition and treatment.

In this case study, we present a young infant with a life threatening early symptomatic congenital syphilis, whose mother tested negative for syphilis in the antenatal screening at sixteen weeks of pregnancy. Congenital syphilis should be considered in the differential diagnosis of a sick infant presenting with an infection and undefined skin abnormalities. It is important to realize that congenital syphilis can still occur in developed countries with a high coverage rate of antenatal screening as *T. pallidum* spirochetes can be transmitted between testing in pregnancy and labor. Indeed, in our case, congenital syphilis was initially unrecognized by all involved physicians of the parents and their infant. We provide a review of the literature on epidemiology, transmission and clinical presentation and subsequently discuss the key messages of this case.

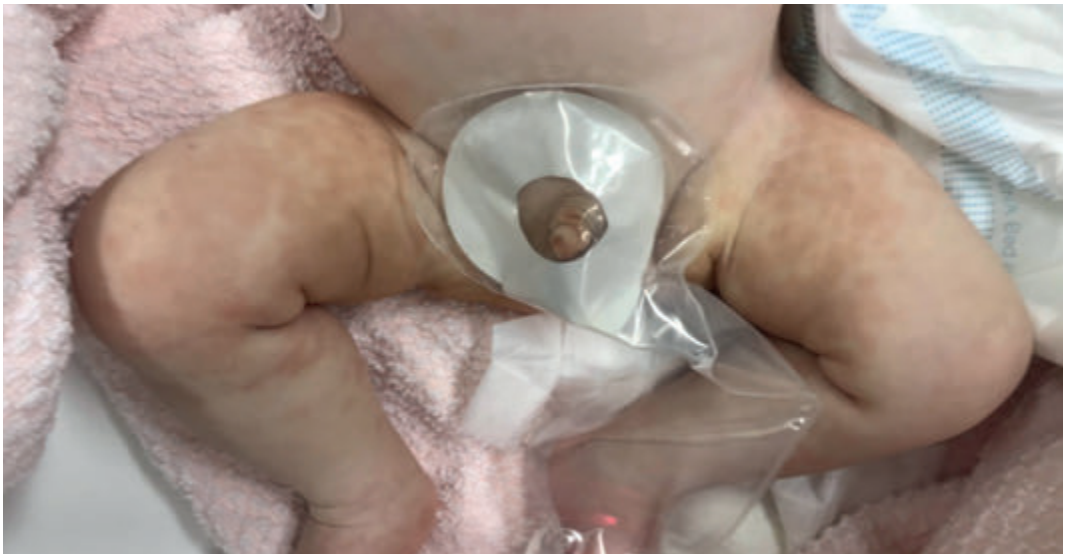


Figure 1 Skin lesions of the patient at presentation

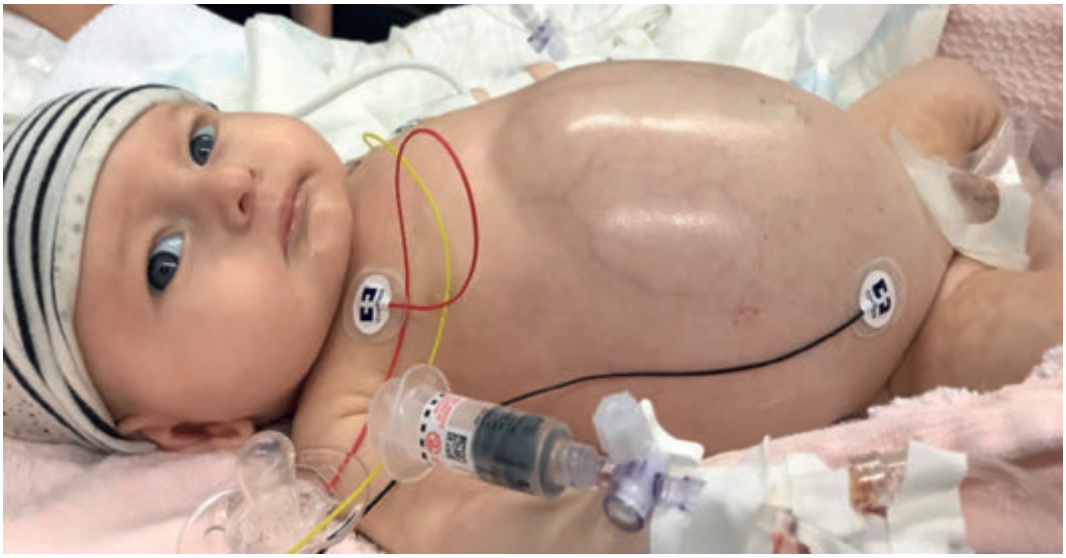


Figure 2 Clinical picture at presentation

CASE PRESENTATION

In December 2018, a three months old boy was presented at the emergency room in the province of Utrecht in the Netherlands with an undefined rash, progressive abdominal distension and hypothermia. He was born after 38 weeks of gestation with a birth weight of 2880 grams. The mother had received all antenatal pregnancy screening, testing negative for syphilis antibodies at 16 weeks of gestation. The pregnancy, vaginal delivery, and neonatal period were uneventful. The family was of middle-income class, originated from the Netherlands and living in a small town in the province of Utrecht. The medical history reported skin lesions around three weeks prior to presentation, followed by several days of low grade fever, loose stools and mild but progressive feeding problems.

The rash, initially located on the face spreading to the chest and legs, consisted of reddish-brown colored lesions of 1 cm (figure 1). Ten days prior to presentation, a consulted dermatologist interpreted these lesions as seborrheic dermatitis or viral exanthema. Progressive abdominal distension was noticed and progressed dramatically during three days prior to presentation. On admission, the patient was alert, the temperature was 37.8 °C and vital signs were within normal ranges. Physical examination showed a pale and grunting boy, the abdomen was largely distended with spider naevi and diffuse peripheral edema (figure 2).

Laboratory findings are reported in table 1. Complete blood count showed severe anemia (2.6 mmol/l) and thrombocytopenia (13 x10^9/l) with elevated leucocytes (43.8 x10^9/l). Microscopic examination of a stained blood smear did not show any lymphoblasts. Moreover, coagulation parameters and liver enzymes were grossly disturbed. Abdominal ultrasound showed extensive ascites and hepatomegaly with normal anatomy of liver, gallbladder and bile ducts. Subsequently, intravenous fluids, blood transfusion and empirical treatment with ceftriaxone 500 mg were administered. In the following hours, the patient was transferred to an intensive care unit due to increasing respiratory, renal and liver failure with signs of portal hypertension and preparations were made for a liver transplantation.

Laboratory results		Ref. value			Ref. value
Hemoglobin	2.6 mmol/l	6.0-11.0	ALAT	88 U/l	<45
Leucocytes	43.8 x10^9/l	5.0-20.0	ASAT	181 U/l	<80
- Neutrophils	17 x10^9/l	1.5-10			
- Lymphocytes	24 x10^9/l	3.0-14			
- Monocytes	3.1 x10^9/l	0.0-2.0			
- Eosinophils	<0.1 x10^9/l	0.0-2.0			
- Basophils	<0.1 x10^9/l	0.0-0.2			
Platelets	13 x10^9/l	140-450	GGT	579 U/l	<200
CRP	69 mg/l	<5	PTT	17 sec	9.9-11.8
Lactate	4.7 mmol/l	<2.2	APTT	55 sec	22-28
Sodium	117 mmol/l	137-144	Albumin	15 g/l	35-50
Potassium	6.6 mmol/l	3.5-5.0	Uric acid	0.3 mmol/l	0.20-0.42
Creatinine	13 umol/l	<115	LD	396 U/l	<625

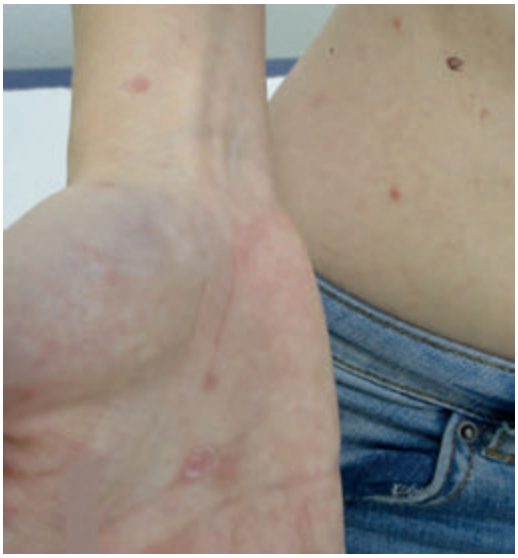
Table 1 Laboratory values at presentation
Abbreviations: ALAT, alanine aminotransferase; APTT, activated partial thromboplastin time; ASAT, aspartate aminotransferase; CRP, C-reactive protein; GGT, gamma glutamyl transferase; LD, lactate dehydrogenase; PTT, prothrombin time; ref. value, reference value; sec, seconds.

During the next week the bacterial cultures of blood, stools, urine and cerebral spinal fluid (CSF) were negative. Polymerase chain reaction (PCR) assay for HIV antigen, hepatitis A, B, C and E, herpes simplex types 1 and 2, adeno-, cytomegalo-, Epstein Barr-, entero-, and human parecho virus in blood and/or CSF and PCR for respiratory viruses in mucosal samples were also negative. There were decreased levels and activity of complement C3 and C4, decreased IgG and elevated IgM levels. Whole exome sequencing did not show any clues of primary immunodeficiency or complement deficiency, thus the decreased complement factors were presumably caused by increased use following infection. Further immunological assessment including alfa1-antitrypsine activity, anti–liver-kidney microsomal antibodies, antinuclear antibodies, and anti-neutrophil cytoplasmic antibodies, was normal. Metabolic screening of blood and urine reported no abnormalities. Furthermore, histological examination of the skin lesions showed non-specific superficial infiltration. Bone marrow biopsy showed no signs of leukemia. By abdominal magnetic resonance imaging and lip biopsy, gestational alloimmune liver disease was excluded. A liver biopsy was not possible due to the coagulation disorder and the amount of ascites.

In the absence of a diagnosis, therapeutic ascites punctures were performed and cefotaxim, metronidazole, acyclovir, dexamethasone, and intravenous immunoglobulins were administered. The liver function and hematologic abnormalities improved slowly a week after first admission and normalized in the following two months, unexpectedly. Gradually, the patient recovered completely, including normalization of all laboratory abnormalities, and he was discharged after a total of 42 days admission. Up to date, the patient is still in excellent health acquiring the normal pediatric milestones.

Ten weeks after the initial presentation, the father reported to his general physician having a throat ache and skin lesions located on the scrotum. Upon blood testing of the father, positive treponemal tests were found with a rapid plasma reagin (RPR) titer of 1:64, indicating an active syphilis infection. Family history of the father revealed contact with a possible but not confirmed syphilis patient at around 25 weeks of gestation. Two months later, the father presented in the emergency room due to fever, headache and signs of an otitis for which he was treated with oral amoxicillin and his complaints resided. Further family history revealed that the mother also presented with similar lesions four weeks prior to the symptoms of the patient (figure 3). She had consulted an immunologist and dermatologist and was diagnosed with lichen planus via a skin biopsy.

Figure 3 Skin lesions of the mother



During her regular antenatal screening, she was tested negative for syphilis antibodies at 16 weeks of gestation. After a confirmed diagnosis of the father, the mother was tested positive in treponemal testing, with an RPR titer of 1:32, confirming a recent active syphilis infection. Revision of her skin biopsy with immunohistochemical antibody staining showed multiple spirochetes in the epidermis.

Retrospective testing of the infant's stored serum, collected on admission, revealed an RPR titer of 1:64 and a positive fluorescent Treponema antibody absorption (FTA-ABS) test. The diagnosis of congenital syphilis was confirmed with a positive *T. pallidum* IgM immunoblot, performed at the national reference center (National Institute of Public Health and the Environment, Bilthoven, The Netherlands). Longitudinal RPR titers decreased from 1:64 to 1:4 in the following two months without additional treatment. Both parents were treated with penicillin, with direct clinical improvement of their general condition. A timeline of clinical presentation and syphilis testing of father, mother and the patient is displayed in figure 4.

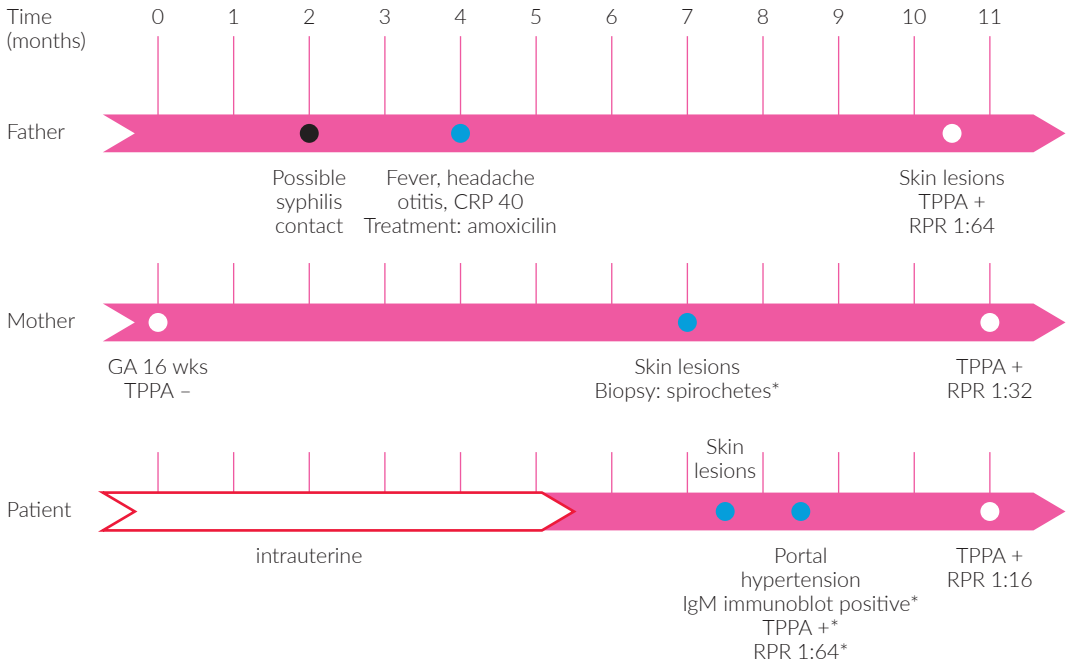


Figure 4 Timeline of the patient and parents

Time in months, with t=0 at 16 weeks pregnancy. * Retrospective testing at t=11 of taken samples. + positive result, - negative result. Abbreviations: CRP, C-reactive protein; GA, gestational age; IgM immunoblot; ***Treponema pallidum*** Immunoglobulin M immunoblot; RPR, rapid plasma reagin titer; TPPA, ***Treponema pallidum*** particle agglutination; wks, weeks.

REVIEW AND DISCUSSION

In this Grand Round we describe a severe life threatening case of congenital syphilis. This case demonstrates the difficulty in recognizing syphilis by its nonspecific skin lesions in both parents and their child. Syphilis, what is often thought of as a forgotten disease in developed countries, may not have been considered as the diagnosis by many physicians involved due to a perceived low risk for infection. This case illustrates that a negative syphilis test during pregnancy does not rule out congenital syphilis, as intercurrent transmission of *T. pallidum* can still occur between testing in pregnancy and labor.

EPIDEMIOLOGY

Since 2007, the antenatal screening program of the WHO is targeting the global elimination of mother-to-child *T. pallidum* transmission and congenital syphilis.[8] Global maternal syphilis prevalence was estimated at 690 per 100,000 in 2016.[9] The global rates for congenital syphilis was 473 per 100,000 live births in 2016 with a total of 355,000 adverse birth outcomes and 306,000 neonates born to a syphilis seropositive mother with no or inadequate treatment. Included in these high incidence rates are adverse birth outcomes with 143,000 fetal deaths and still births, 61,000 neonatal deaths, 41,000 preterm of low-birth weight births and 109,000 infants with clinical disease, confirming the burden of disease worldwide.[9]

Some countries, such as Cuba and Thailand, have achieved elimination of mother-to-child syphilis transmission, meaning low congenital case rates and high coverage of antenatal screening and treatment of infected pregnant women.[4] Although syphilis infection is still limited in developed as compared to developing countries, a steady in-

crease has been reported in the United States with an overall rate of congenital syphilis cases of 8.4 per 100,000 live births in 2012 to 33.1 cases per 100,000 in 2018.[6] Despite the efforts of preventative antenatal care, this represents a 40% increase from the numbers identified in 2017.[10] This higher incidence reflects the increase in the rate of primary and secondary syphilis infection in women of reproductive age.[6] Most cases (even in developed countries) are due to delayed or no screening of pregnant women and late or inadequate treatment. [7]

The Dutch antenatal screening detected 97 to 135 cases of syphilis seropositive pregnant women yearly, providing an estimated prevalence of 60 to 80 per 100,000 in recent years (2012-2016).[7] As some of these cases represent women with endemic treponematoses or already treated or spontaneously resolved cases, the true prevalence of syphilis infected pregnant women is expected to be even lower. The rate of registered cases of congenital syphilis among Dutch children in 2009-2015 varied between 0.00–3.33 cases per 100,000 live births.[7] True rates of syphilis infection in pregnant women and infants, however, may be different as syphilis is not a notifiable disease in the Netherlands and causes of death for stillbirths are not registered. This lack of data is one of the reasons why the Netherlands has not been validated for elimination of mother-to-child syphilis transmission. As the worldwide incidence of syphilis increases, even in developed countries such as the United States, adequate antenatal screening programs and case reporting of congenital syphilis are crucial in the elimination activities of the WHO.[8]

MOTHER-TO-CHILD TRANSMISSION

Congenital infection is caused by transplacental transmission during pregnancy or, less commonly, through contact with maternal infected skin lesions during birth. Mother-to-child transmission can occur at any gestational age and transmission rates are highest in primary and secondary stages of maternal syphilis infection (40-90%) compared to latent disease (respectively 40% and <10% in early and late latent disease).[11] All syphilis seropositive pregnant women should be treated with penicillin to prevent transmission regardless of stage of disease or gestation.[11] Partner notification and treatment of seropositive women is a crucial element of preventative care. Moreover, additional testing for maternal coinfection of other sexually transmitted diseases, such as HIV and hepatitis B virus, should be performed. The WHO criteria for elimination of syphilis transmission indicates a required antenatal care coverage (at least one visit) of ≥ 95%.

In the Netherlands, all pregnant women are counselled to be screened for hepatitis B, HIV, syphilis, blood grouping and red cell antibodies during their first antenatal visit, e.g. before the 13th week of gestation.[12] If a positive result is found, further confirmation tests are done and the patient is referred to secondary care for treatment and follow-up. If the antenatal screening is negative, no further tests for syphilis are implied. Known risk groups for syphilis infection may warrant evaluation for (re)infection later in pregnancy. These women should be informed frequently, and laboratory testing can be repeated if the pregnant woman is considered to be at high risk. Repeated testing is not included in standard antenatal care in the Netherlands. In contrast, some other countries, as the United Stated guidelines recommend repeat screening during the 3rd trimester for high risk groups.[13]
A recent study evaluating the WHO validation criteria for elimination of mother-to-child transmission in the Netherlands reported a high antenatal screening coverage for syphilis of more than 99% currently.[7] Unfortunately, adequate data is missing as registration of congenital syphilis cases and of pregnant women treated for an active syphilis infection is not imbedded in the national screening program. Considering the increases reported even in developed countries, awareness and registration is needed in the progress towards elimination of mother-to-child syphilis transmission.

Several risk factors for mother-to-child transmission have been identified, with higher transmission rates in pregnant women with increased RPR titers, with a diagnosis at gestational age > 36 weeks compared to ≤ 12 weeks (adjusted odds ratio 25.0, P 0.001) and if treatment of the mother was < 4 weeks before delivery.[14, 15] Socioeconomic factors as well as access to timely and adequate antenatal screening greatly influence incidence of congenital syphilis in developing and developed countries.[16, 17]

NONSPECIFIC CLINICAL PRESENTATION, A POTENTIAL RISK OF LATE DIAGNOSIS

Recognition of congenital syphilis is difficult as clinical presentation is diverse and often nonspecific. Moreover, as syphilis is mostly known as a sexually transmitted disease in adults, the diagnosis of congenital syphilis in children is often not considered. Depending on the time of transmission, a congenital syphilis infection can cause several ante- and perinatal problems, including stillbirth, prematurity and low birth weight. More than half of live-born infants with congenital syphilis are asymptomatic at birth, with first clinical signs presenting by the age of three months. In early congenital syphilis, defined by an onset of symptoms before two years of age, common signs include vesicular or maculopapular rash, failure to thrive, hepatomegaly, generalized lymphadenopathy and fever. However, many organ systems can be affected, causing i.a. hematological, neurological and skeletal abnormalities (see ref for detailed summery).[18] Cutaneous signs can variate from a macular eruption to condylomata lata or roseolae, causing difficulty in early recognition. In a cohort of fifty neonates with early symptomatic syphilis, although diagnosed and treated in South Africa, 68% required intensive care and mortality rate was 38%.[19] In late congenital syphilis, clinical signs can be more subtle and become apparent years later, such as interstitial keratitis, deafness and dental abnormalities (known as Hutchinson's triad), neurological or musculoskeletal problems as the infant grows older.[11] Early initiation of treatment can prevent development of these late effects of disease. Also, rebound infection within families through nasal secretions or skin lesions in early stages of untreated disease should be prevented.

DIAGNOSTIC TESTS

A diagnosis is confirmed with *T. pallidum* spirochetes through darkfield microscopy of lesions or body fluids, a PCR positive result, immunohistochemistry or special stains of specimens from lesions or other samples.[6] However, PCR testing is not available in all laboratories and microscopic recognition of syphilis bacteria can be difficult. The cornerstone in diagnostics is still serology: the currently used diagnostic algorithm in the Netherlands combines nontreponemal- and treponemal specific tests. The combination of these tests increases accuracy as other medical conditions can cause false-positive results and treponemal testing can remain positive even though the infection has been treated adequately.[11]

Nontreponemal tests include the Veneral Disease Research Laboratory (VDRL) slide test and the RPR test and measure levels of total Ig antibodies against lipoidal antigens from damaged host cells. Congenital syphilis is notoriously difficult to diagnose with the current available diagnostic testing. Generally, titers in the infant should be at least fourfold higher postpartum than the maternal titers to represent congenital disease. Demonstration of IgM *T. pallidum* antibodies in the infants blood by immunoblotting is currently the most sensitive serologic method for evidence of congenital *T. pallidum* infection.[15] Methods and definitions tend to differ internationally. While some countries rely on an IgM of fourfold increase in titers to diagnose congenital syphilis, there are different criteria for diagnosis followed elsewhere, identifying cases through physical exam findings or CSF evaluation also.[11]

Nontreponemal tests are used to monitor activity of disease and response to therapy as they provide quantitative results and titers decline rapidly after adequate treatment. In this case, the RPR titers in the patient decreased from 1:64 to 1:4 during follow-up without additional treatment. Most likely, the RPR titers decreased by the empirical antibiotic treatment the patient received upon admission. Treponemal testing detects treponemal specific antibodies through *T. pallidum* haemagglutination or particle agglutination assay (TPHA/TPPA), or more recently through enzyme immune assays (EIA) . In these tests, surface antigens of *T. pallidum* are combined with serum of the patient to demonstrate reaction with specific antibodies. Although treponemal tests are considered more specific than nontreponemal tests, false-positive results can still occur (e.g. in *Borrelia burgdorferi* infections or other non-veneral treponematoses). Therefore, positive treponemal screening tests are being confirmed in the FTA-ABS or in a *T. pallidum* immunoblot.[20] As these methods detect total immunglobuline antibodies to *T. pallidum*, they do not distinguish between passively transferred antibodies from the mother and antibodies of the infant following congenital infection. Despite the mentioned challenges of testing, treatment should be provided to any infant in whom there is a clinical suspicion of syphilis (regardless of certainty of the diagnosis based on serology).

This approach of treatment, is justified given the low risk of treatment and high risk of long term morbidities associated with untreated disease.

CASE DISCUSSION

The rising global incidence rates - even in developed countries - are striking, thus recognition of congenital syphilis is important. Fortunately, high coverage of antenatal screening has kept the congenital syphilis case rates relatively low in the Netherlands over the last years. This case illustrates that due to these low (congenital) syphilis rates, a syphilis infection may not be considered in a physician's differential diagnosis in families with skin lesions and nonspecific symptoms such as low grade fever. It further underlines the importance of the family history, which made a communicable disease more likely and finally provided the clue to the diagnosis. Even more so, congenital syphilis could be overlooked in a general population including pregnant woman adequately attending antenatal screening, without known risk factors for sexually transmitted disease.

Several missed opportunities of (early) diagnosis can be recognized in this severe congenital syphilis case. Some weeks prior to presentation in the emergency room, both skin lesions of the mother and the patient have been assessed by dermatologists and a skin biopsy had been investigated. However, if syphilis is not considered in the differential diagnosis and not actively requested for, a skin biopsy will fail to identify *T. pallidum* spirochetes. Based on a visual evaluation of the rash, other diagnoses such as seborrheic eczema and lichen planus were considered more likely in this family. As the lesions of early syphilis - also known as the great imitator - have often been mistaken for those of other infections, early diagnosis may be hampered.[21]

Although these skin abnormalities could have been suggestive in an earlier stage, the first manifestations of congenital syphilis often includes nonspecific symptoms such as feeding problems, failure to thrive and low grade fever.[18] Besides these nonspecific symptoms, the variety of congenital syphilis manifestations is known for mimicking other diseases. Similar to our case, patients have been described presenting with hematological manifestations predominantly.[22] While these hematological abnormalities such as hemolytic anemia or thrombocytopenia are common in congenital syphilis, they can very well be suggestive for a malignant infiltrative or proliferative bone marrow disorder. Other diagnoses are often considered first, even more so in countries with low syphilis rates. Despite the low incidence all physicians who care for pregnant women and infants should be aware of the manifestations of syphilis.

Finally, physicians should be aware of the fact that syphilis can be acquired at a later stage in pregnancy after antenatal screening has been performed. An interesting point of this case is the fact that the mother had received adequate screening in antenatal care, including syphilis. Most cases of congenital syphilis are the result of missed screening or inadequate follow-up after positive test results.[6] Moreover, all registered cases of congenital syphilis in 1997-2008 in the Netherlands were the result of infected mothers of vulnerable groups, such as illegal immigrants or drug-users, who had withdrawn from antenatal care.[23] This Caucasian family was of middle-income class without any known risk factors for sexually transmitted infections, such as drug use, sex work or immigrant background. The mother was not screened more often than the recommendation of the Dutch national antenatal screening protocol. An extra screening test in this mother in the third trimester could have prevented transmission. Considering the low prevalence of syphilis in the regular population, screening at more time points during pregnancy would probably not be a cost-effective strategy in the elimination of congenital syphilis. However, even in countries with low prevalence, women of high risk should be counselled about the importance of safe sex and repeat antenatal screening. And most importantly, this case underlines that negative syphilis screening results in early pregnancy do not rule out the possibility of a congenital syphilis infection.

CONCLUSION

The case presented in this Grand Round demonstrates that transmission of *T. pallidum* can still occur in developed countries with a high coverage rate of first trimester screening. The broad spectrum of clinical manifestations in congenital syphilis infection, mimicking many alternative diagnoses, may lead to diagnostic delay with severe consequences. Specifically, this preventable infectious disease may easily be overlooked if acquired late in pregnancy

and thus occurring after antenatal screening. Thus, congenital syphilis should be considered in any severe and diagnostically challenging infectious disease case even in the context of negative antenatal screening.

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Chapter III

Inconsistent management of neonatal herpes simplex virus infections

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ABSTRACT

Objective

The incidence of neonatal herpes simplex virus (nHSV) infections is monitored periodically in the Netherlands, yet management and outcome is unknown. Comprehensive national guidelines are lacking. We aim to describe management and outcome in the last decade to explore current diagnostic and therapeutic challenges. We aim to identify possible variability in management of patients suspected for nHSV infection.

Methods

We conducted a retrospective case-series of management and outcome of nHSV infections at two tertiary care center locations in the Netherlands.

Results

A nHSV infection was diagnosed in 1% (12/1348 patients) in whom polymerase chain reaction for HSV was performed. Of the nHSV patients, 3/12 died and 4/9 (44%) survivors suffered neurological sequelae. Neurological symptoms at presentation were seen in only 2/8 nHSV encephalitis cases. Cerebral spinal fluid analysis was performed in 3/6 patients presenting with skin lesions. Only 3/6 neurological cases received suppressive therapy. nHSV infection was diagnosed in 8/189 (4%) empirically treated patients.

Conclusions

Management of nHSV infection, particularly when presenting with skin lesions, is inconsistent. Many infants without a HSV infection are exposed to antiviral medication. There is substantial inter-hospital variation in diagnostic and therapeutic management of a suspected infection. Comprehensive guidelines need to be developed to standardize management of suspected nHSV infection.

INTRODUCTION

The incidence of neonatal herpes simplex virus (nHSV) infections, although low, has increased over the past fifteen years in the Netherlands.[1] This problem is emerging since the HSV seroprevalence among adults is declining, resulting in a higher number of unprotected women of reproductive age thus increasing the risk of nHSV disease.[2] The course of disease ranges from skin lesions to severe neurological disease or multi-organ failure.[3] In progressed infection, substantial mortality and severe morbidity due to neurological sequelae have been described.[4]

There are several difficulties in recognizing nHSV infection as > 80% of maternal viral shedding is asymptomatic and initial presentation is often nonspecific.[5, 6] International guidelines state contrasting recommendations and, in many countries, comprehensive national guidelines are lacking.[7, 8] In 2011, a trial showed beneficial effects of prolonged six months treatment for proven neurological nHSV infection.[4] However, there is no consensus on what diagnostic testing is required in infants with a suspected nHSV infection to identify patients with indication for prolonged treatment.

We aim to describe outcome and current management challenges and inconsistencies of nHSV infections in the Netherlands. Identification of these challenges will support the development of a future (inter)national guideline. We aim to identify possible variability in the diagnostic and therapeutic approach of patients suspected for nHSV infection.

MATERIALS AND METHODS

We performed a chart review of all nHSV cases admitted to two tertiary care centers in the northern region of the Netherlands, from 2006 until 2017. We identified patients aged ≤ 60 days suspected of a nHSV infection (i.e. sample collection for HSV polymerase chain reaction (PCR) testing) through a patient database query. We collected the following data: clinical data, HSV PCR on any material, cerebral imaging results, residual symptoms, recurrent infections, developmental delay. If available, Bayley Scale of Infant Development (BSID) scores or Gross Motor Function Classification System (GMFCS) scores were documented. The BSID scores mental and motor development (range 50-150, mean of 100). The GMFCS categorizes impairment in gross motor function of children with cerebral palsy (range level 1- 5).

We defined nHSV disease as a HSV (type 1 or 2) infection PCR-confirmed in a blood, cerebral spinal fluid (CSF) or skin/mucosal sample. PCR testing is performed in both tertiary care centers and is considered the primary diagnostic test in the Netherlands. Patients were categorized into three groups[3]: skin, eye, mouth (SEM), central nervous system (CNS) and disseminated (DISS) disease. SEM disease is limited to vesicular SEM lesions with a PCR-positive skin/mucosal sample. CNS disease or HSV encephalitis is characterized by neurological involvement with 1) a PCR-positive CSF sample despite neurological signs (seizures, hemiparesis, lethargy, abnormal neuroimaging, CSF abnormalities) or 2) clinical signs of CNS involvement with a PCR-positive blood or skin sample. In DISS disease signs of multi-organ involvement are present with any HSV PCR-positive sample. Adequate therapy was defined as intravenous (IV) acyclovir 60 mg/kg/d during 14 or 21 days for SEM and CNS/DISS disease respectively.[3] Percentages are rounded to the nearest whole number. This study was approved by the Medical Ethics Review Committee and a waiver for the Medical Research Involving Human Subjects Act was provided.

RESULTS

A total of 1% of patients (12/1348) evaluated for a nHSV infection following a clinical suspicion were included in the study with PCR-confirmed nHSV disease (8/1120 patients at the first center and 4/228 at the second center) (figure 1). In 4% of patients (8/189) started on empiric acyclovir, a diagnosis with nHSV disease was confirmed (5/72 patients at the first center and 3/117 at the second center).

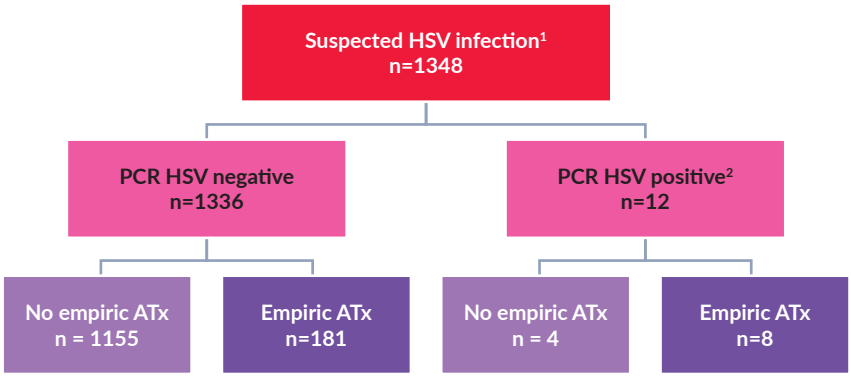

Figure 1 Patient inclusions

[1] PCR for HSV performed on any sample of blood, cerebral spinal fluid or skin/mucosal lesions of suspected patients

[2] ≥1 positive PCR result on any sample

ATx, intravenous acyclovir therapy.

Table 1 displays clinical course and management of the cases: 8/12 categorized as CNS disease, 3/12 as SEM disease and 1/12 as DISS disease. Age at the onset of symptoms ranged from 0-30 days, with 5/12 patients presenting first symptoms at age 2-3 weeks. In total, 6/12 patients presented with SEM lesions and only 2/12 with fever. Of all CNS disease cases, 2/8 presented with seizures, while the remaining 6 cases presented with lesions or a subfebrile temperature. Of the CNS patient without neurological presentation, 3/6 developed neurological symptoms during admission, while 3 patients did not have neurological symptoms despite a PCR-positive CSF or MRI abnormalities. Delay in testing ranged from 0-12 days and counted ≥ 2 days in 5/12 cases. All cases with delay in testing presented with nonspecific symptoms (irritability, poor feeding). In two cases testing was delayed several days after presentation due to misdiagnosis of SEM lesions. Cerebral MRI was performed in 7/8 CNS cases, all showing hemorrhage or diffusion restriction in cortical parts of the brain. Two out of eight CNS patients did not survive the infection. The only DISS patient, who was started on acyclovir after a delay of seven days, deceased. Of the surviving HSV cases, 4/9 (44%) developed neurological sequelae in the following years (follow-up time 1-12 years). Upon discharge, there were no residual symptoms in most of the infants who developed sequelae. History of maternal HSV was reported in seven cases. Genital lesions during the last trimester were reported and treated in two cases.

	Year	HSV class	Co-morbidity	Presenting symptoms	HSV contact	HSV + sample	Age at symptom onset
1	2017	CNS HSV-2		T 37.9 °C	No	CSF	16
2	2017	CNS HSV-2		T 37.8 °C	Mat Oth	CSF, plasma	16
3	2017	CNS HSV-1		lesions	Mat	CSF	16
4	2017	CNS		T 38.2 °C seizures	No	CSF	?
5	2016	SEM		lesions T 37.0 °C	Oth	skin	10
6	2014	CNS HSV-2	prem 27 wk	lesions	Mat	CSF, plasma	0
7	2013	SEM		lesions T 36.8 °C	No	plasma	9
8	2010	CNS HSV-1	SGA	none	Mat	CSF, sputum	-
9	2007	SEM		lesions T 37.0 °C	Mat	skin	18
10	2006	CNS		seizures	Oth	CSF, plasma	17
11	2006	CNS HSV-2	prem 31 wk	lesions T 37.2 °C	Mat Oth	skin, ocular	30
12	2006	DISS		T 39.0 °C	Mat	plasma	5

Table 1 Characteristics, management and outcome of neonatal HSV infection cases
Abbreviations: AEDs, Anti-epileptic drugs; BSID, Bayley Scale of Infant Development (scores ranging from 50-150, mean of 100); CNS, central nervous system disease; CSF, cerebral spinal fluid; DISS, disseminated disease; GMFCS, Gross Motor Function Classification System (scores ranging from level 1 (no impairment) to level 5 (severe impairment); HSV, herpes simplex virus; Mat, maternal; Oth, other; prem, prematurity; Recurr, recurrence of HSV infection ; SEM, skin eye mouth disease; SGA, small for gestational age; Supp, suppressive therapy; T, temperature in degrees Celsius; Tx, acyclovir therapy; wk, weeks.

Age at sample collection	Age at Tx start	Tx length	Outcome	Supp	Sequelae after discharge	Recurr	BSID/ GMFCS
17	17	4	deceased	-	-	-	-
18	18	4	deceased	-	-	-	-
28	28	21	recovery	Yes	No	3x SEM	?
38	38	21	recovery	No	abnormal moving pattern on AEDs	No	?
12	12	14	recovery	No	No	2x SEM	?
0	3	14	recovery	Yes	hemiparesis	>3x SEM	BSID 94 GMFCS 1
12	12	21	recovery	No	No	No	?
1	4	21	recovery	No	No	No	?
18	20	14	recovery	No	No	No	?
17	17	21	recovery	No	diplegia	No	?
30	34	7	recovery	Yes	epilepsy, on AEDs PM retardation vision impaired	>3x SEM	GMFCS 5
13	13	4	deceased	-	-	-	-

HSV PCR testing on CSF was only performed in 3/6 (50%) patients presenting with SEM lesions. In this group, HSV PCR testing was performed on blood samples in 3/6 and on ocular/skin samples in 3/6 patients. Delay from onset of symptoms to initiation of adequate treatment was ≥ two days in this group. Adequate therapy was not initiated (according to treatment guidelines) 4 in 3/12 cases, that were all patients presenting with SEM lesions. In case six acyclovir was discontinued after fourteen days despite neurological involvement and in case seven the patient was switched to oral valacyclovir, after three days due to a mild clinical course. In case eleven the patient presented with HSV PCR positive SEM lesions and initial treatment consisted of topical acyclovir cream IV acyclovir was added after four days due to CSF pleocytosis and stopped after seven days when HSV PCR in CSF was negative. This particular patient suffered neurological sequelae and recurrent HSV infection. Only in one CNS case CSF was checked and negative before ending therapy.

Suppressive therapy was administered in 3/6 (50%) surviving CNS cases. In one case suppressive therapy was given for two years due to recurrence, after which acyclovir resistance developed. In another case suppressive therapy was continued for four years due to recurrent SEM disease. The three remaining CNS cases surviving initial infection have not been treated with suppressive therapy, two of which developed late sequelae.

DISCUSSION

Our results show that mortality and morbidity rates of nHSV disease, CNS disease in particular, are high. HSV CNS disease often presents without neurological symptoms. Also, we found substantial inter-hospital variation in diagnostic and therapeutic management of suspected nHSV infection, particularly in patients presenting with skin lesions. The incidence rate is in line with the latest Dutch monitoring study reporting an incidence of 4.8 per

100,000 births.[1] Comparable morbidity rates have been described in earlier studies reporting normal development in only 30-35% of CNS disease patients.[4, 9]

Moreover, we found a nonspecific presentation to be common, such as nHSV CNS cases without neurological signs at presentation or during admission. A nonspecific presentation is likely to lead to variation in management and treatment delay in the absence of uniform guidelines. Practice variation was particularly substantial in PCR testing of patients presenting with SEM lesions. Reluctance to perform CSF analysis is understandable given the invasive nature of a lumbar puncture and the rarity of the disease. Yet without CSF analysis a proportion of nHSV CNS cases remain unrecognized and antiviral medication will be incorrectly withheld. Inadequate treatment of neurologically involved patients often leads to lifelong disabilities and increased healthcare costs.[4] Additionally, end-of-treatment reassessment of CSF HSV PCR was rarely performed. Despite the international opinion that neurological involvement cannot be excluded based on clinical presentation, not all guidelines state clearly when CSF analysis is indicated to rule out neurological infection or to confirm treatment effect.[7]

The most important dilemma remains what characteristics should prompt a clinician to perform PCR testing and which justify empiric acyclovir. Despite reported adverse effects of acyclovir some experts suggest a low threshold to empiric treatment.[10] All proven nHSV cases from our cohort identified from 2016 forward (cases 1-5) were treated on the same day as testing was performed, whereas 4 of 7 cases (6-12) before 2016 had treatment initiated at least 2 days after testing. Gaensbauer et al. suggest a tendency towards increasing empiric acyclovir treatment in HSV negative cases beyond the neonatal age in more recent years.[11] However, the risks of over-treating infants should be taken into consideration in low incidence countries and cost-effective benefits of empiric treatment in case of suspected nHSV infection has not been proven. As our study was not designed to address tendencies in empiric acyclovir treatment in the HSV negative neonates, this might be an interesting objective for future research. Ours and earlier studies show inter-hospital variation in diagnostic and therapeutic approach to suspected nHSV infection in the Netherlands.[12] This reflects the lack of national consensus, which has also been underlined in other countries.[13, 14] The American Academy of Pediatrics gives some practical guidance on PCR testing, although precise testing and treatment indications are lacking.[3] The Dutch guideline on febrile infants advises to only treat empirically in case of neurological symptoms based.[15] This is in contrast to the Canadian guidelines stating empiric acyclovir in all unwell infants under six weeks suspected for sepsis.[7] Additional characteristics to prompt empiric therapy have been mentioned, such as vesicular lesions or pleocytosis.[12, 13] As prospective trials are limited by the low incidence and required follow-up time, larger-scale observational studies should focus on the value of clinical characteristics in identifying patients requiring CSF testing and empiric therapy to develop evidence-based guidelines.

This study is limited by its retrospective nature and completeness of data was dependent on medical records as there is no national registry on long term outcomes available in the Netherlands. Prospective studies on nHSV disease are limited due to the low incidence and the required follow-up time. Although it is likely that follow-up would be conducted at the treating tertiary care centers, cases of long-term sequelae could have been missed. Also, follow-up time differs between cases and further disabilities might become apparent in the future. Although a small sample size was inevitable, it prevented us from analyzing differences between subgroups. We tried to minimize the selection bias by identifying infants through a query of both patients in whom HSV PCR was performed and patients started on acyclovir. The search in the pharmacy database was merely done to decrease the chance of missing potential inclusions due to incomplete reporting. All patients treated with acyclovir were PCR tested.
Our data provides insight in the clinical course and outcome of nHSV disease. This case series underlines the current management challenges and the need for uniform national guidelines in order to improve recognition and management of suspected nHSV infection.

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Chapter IV

Comorbidities, clinical characteristics and outcomes of COVID-19 in pediatric patients in a tertiary medical center in the Netherlands

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Having pre-existing comorbidities is described as a risk factor for more severe disease in adult COVID-19 and in infections with SARS-CoV-1 and MERS-CoV.[1] In adult SARS-CoV-2 infections, patients with pre-existing underlying comorbidities, such as chronic obstructive pulmonary disease, cardiovascular disease, diabetes and obesity, are more likely to have severe disease compared to healthy adults.[2] An inconsistency is seen in current findings on the association with comorbidities and pediatric COVID-19 severity. An important limitation in currently available studies is limited data: severe disease is rare in children compared to adults, and most studies describe COVID-19 severity merely by reporting intensive care unit (ICU) admission or mortality rates instead of detailed data on clinical presentation and outcomes.

A severe manifestation of SARS-CoV-2 infection is multi inflammatory syndrome in children (MIS-C), which usually follows weeks after SARS-CoV-2 infection and is characterized by gastrointestinal symptoms, muco-cutaneous signs and cardiovascular involvement. Several studies describe the association between comorbidities and incidence or mortality of MIS-C.[3, 4] To our knowledge, there are no data on the association between pre-existing comorbidities and the severity of MIS-C.

Thus, the primary aim of this retrospective study was to describe in detail the pre-existing comorbidities and the severity of SARS-CoV-2 infections in pediatric patients in a tertiary medical center in the Netherlands. Second, we aimed to assess the association between comorbidities and disease severity of both acute COVID-19 and MIS-C in pediatric patients. These data will help to determine which groups of children are more vulnerable to severe acute COVID-19 and severe MIS-C, which could aid development of clinical SARS-CoV-2 infection care and management strategies.

This retrospective, observational cohort study was carried out at the tertiary medical center, Amsterdam UMC, the Netherlands. Inclusion criteria were in- and outpatients younger than or equal to 18 years with a positive polymerase chain reaction (PCR) test or serum antibodies (total Ig) against SARS-CoV-2 between March 2020 and April 2021. Patients were excluded when no data on clinical characteristics of the SARS-CoV-2 infection were available. Data describing pre-existing comorbidities, COVID-19 severity and clinical outcomes were retrieved from medical records. Comorbidities were assessed by extracting data on pre-existing disorders based on ICD-10 codes and body mass index (BMI). A pre-existing disorder was found to be relevant when the disorder could potentially interact with the immune system or other bodily functions which could influence disease severity. The pre-existing comorbidities were classified into comorbidity groups based on the affected organ system. We used the definition of childhood obesity using BMI corrected for age and sex in 2000 by Cole et al, to assess the prevalence of obesity for patients aged two years or older.[5]

Disease severity of pediatric acute COVID-19 was classified by Dong et al. into five categories: asymptomatic, mild, moderate, severe and critical.[6] For statistical analyses, asymptomatic, mild and moderate cases were combined as non-severe disease, and severe and critical cases were combined as severe disease. Based on the WHO criteria for MIS-C and parallel to the classification mentioned above, the severity of MIS-C was described in the categories moderate, severe or critical.[6, 7] Moderate cases were classified as non-severe, and severe and critical disease were combined as severe disease for the statistical analysis. Clinical outcome described hospitalization, ICU admission and mortality rates in both acute COVID-19 and MIS-C. Long-term symptoms after acute COVID-19, known as post-COVID-19 syndrome, were reported based on the clinical definition of post- COVID-19 syndrome according to the NICE guidelines[8] ; symptoms that developed during or after acute COVID-19 continuing for more than 12 weeks. Data analysis was performed with Statistical Package for the Social Sciences (SPSS) using Fisher's or Fisher- Freeman- Halton exact tests. A P-value < 0.05 was considered statistically significant. Odds ratios were calculated to describe strengths of associations. In case of contingency tables containing a value of zero, Firth's penalized logistic regression was used to calculate a corresponding odds ratio for the Fisher's exact test to mitigate sparse data bias.[9]

A total of 83 patients were included in this study, among which 46 patients had pre-existing comorbidities. In Table 1, data on demographics and patient characteristics are summarized. Most common pre-existing comorbidities were obesity (n=10, 21.7%), respiratory disorders (n=9, 19.6%) and neurological disorders (n=8, 17.4%). For a detailed description of pre-existing comorbidities in each comorbidity group (supplementary table).

Patient characteristics	**No comorbidities**	**Comorbidities**
	(n = 37)	(n = 46)
Sex, n (%)		
Male	23 (62.2)	23 (50.0)
Female	14 (37.8)	23 (50.0)
Age, y, median (IQR)	11.0 (4.5 – 14.5)	11.5 (4.8 – 16.0)
Weight, kg, median (IQR)	44.3 (26.6 – 57.3)	45.7 (19.5 – 64.0)
Height, cm, median (IQR)	154.0 (122.5 – 168.0)	145.0 (110.0 – 168.0)
BMI, median (IQR)	18.6 (16.6 – 20.7)	18.9 (16.0 – 23.8)
Pre-existing comorbidities, n (%)		
Obesity		10 (21.7)
Respiratory disorder		9 (19.6)
Systemic auto immune disorder		3 (6.5)
Neurological disorder		8 (17.4)
Cardiovascular disorder		5 (10.9)
Endocrine system disorder		4 (8.7)
Hematological disorder		2 (4.3)
Gastrointestinal disorder		3 (6.5)
Urogenital system disorder		6 (13.0)
Genetic/ chromosomal abnormalities		6 (13.0)
Cancer		2 (4.3)
Other comorbidities		4 (8.7)
Pharmacological treatment, n (%)		27 (58.7)
Immunosuppressant medication		7 (15.2)
PCR confirmed SARS-CoV-2 infection, n (%)	22 (59.5)	37 (80.4)
PCR result negative or unknown, n (%)[a]	15 (40.5)	9 (19.6)
Reason for testing, n (%)		
Unknown	5 (13.5)	4 (8.7)
Clinical suspicion of SARS-CoV-2 infection	31 (83.8)	39 (84.8)
Symptoms suspicious for COVID-19	31 (83.8)	33 (71.7)
Contact with COVID-19 case	10 (27.0)	15 (32.6)
Routinely (before procedure)	1 (2.7)	2 (4.3)

Table 1 Patient characteristics and key demographics

[a] Patients with negative or unknown PCR results were included only when (IgM and/or IgG) antibodies against SARS-CoV-2 in serum were present.

From the 58 patients with acute COVID-19, 38 (65.5%) had a pre-existing comorbidity. Most patients had mild COVID-19 disease, in the patient group without comorbidities (n=16, 80.0%) as well as in the patient group with comorbidities (n=24, 63.2%). Eight patients had severe or critical disease (13.8%), all of which had pre-existing comorbidities. One of these patients died due to the consequences of COVID-19. Table 2 summarizes data on severity and hospital admission per group (comorbidities versus no comorbidities). More severe acute COVID-19 was seen in patients with pre-existing comorbidities compared to those without comorbidities (P=0.041, OR 11.42, 95% CI 1.29–1507.49). Patients with a pre-existing comorbidity also had a higher risk of being admitted to the ICU (P=0.032, OR =11.72, 95% CI: 1.31–1547.79) than those without comorbidities.

Outcomes	No comorbidities	Comorbidities
Acute COVID-19, n = 58	**n = 20**	**n = 38**
Severity, n (%)		
Asymptomatic	0 (0)	2 (5.3)
Mild	16 (80.0)	24 (63.2)
Moderate	4 (20.0)	25 (10.5)
Severe	0 (0)	4 (13.2)
Critical	0 (0)	3 (7.9)
Hospital admission, n (%)	7 (35.0)	12 (31.6)
ICU admission, n (%)	0 (0)	6 (15.8)
Hospitalization duration, days, median (IQR)		
Hospital admission	3.0 (0.0 – 6.0)	4.0 (2.0 – 11.0)
ICU admission	0 (0)	2.5 (1.8 – 10.3)
Mortality, n (%)	0 (0)	1 (2.6)
Post COVID syndrome, n (%)	6 (30.0)	2 (5.3)
Fatigue	4 (20.0)	2 (5.3)
Dyspnea	2 (10.0)	
Concentration problems	2 (10.0)	
Dizziness	1	
MIS-C, n = 28	**n = 21**	**n = 7**
Severity, n (%)		
Moderate	7 (33.3)	1 (14.3)
Severe	6 (28.6)	2 (28.6)
Critical	8 (38.1)	4 (57.1)
Hospital admission, n (%)	21 (100.0)	7 (100.0)
ICU admission, n (%)	11 (52.4)	5 (71.4)
Hospitalization duration, days, median (IQR)		
Hospital admission	7.0 (5.0 – 8.0)	7 (7.0 – 10.0)
ICU admission	5.0 (3.0 – 6.0)	3.0 (2.5 – 6.5)
Mortality, n (%)	0(0)	0 (0)
Long term complaints, n (%)	3 (14.3)	1 (14.3)

Table 2 Severity of disease and outcomes

In particular, the presence of a neurological disorder was found to be associated with acute COVID-19 severity (P=0.004, OR 16.11, 95% CI 2.51–103.55). In the group of neurological disorders, 63% had epilepsy or frequent seizures, 50% had cerebral palsy, and other disorders included hydrocephalus and myasthenia gravis. Other main groups of disorders, such as obesity or respiratory disorders, did not show a significant association with disease severity. Table 3 contains the difference in acute COVID-19 severity (non-severe vs severe) between all comorbidity groups and the corresponding P-values.

	Non- severe (n = 50)	Severe (n = 8)	P-value
Sex, n (%)			
Male	29 (58.0)	4 (50.0)	0,715[a]
Female	21 (42.0)	4 (50.0)	
Comorbidities (total), n (%)	30 (60.0)	8 (100.0)	0,041[a,b]
Main groups of pre-existing disorders, n (%)			
Obesity	3 (6.0)	2 (25.0)	0,136[a]
Respiratory system	4 (8.0)	3 (37.5)	0,128[a]
Systemic auto immune disorders	2 (4.0)	-	1,000[a]
Neurological system	3 (6.0)	5 (62.5)	0,004[a,b]
Cardiovascular system	5 (10.0)	-	0,563[a]
Endocrine system	4 (8.0)	-	0,566[a]
Hematological	2 (4.0)	-	1,000[a]
Gastrointestinal system	2 (4.0)	1 (12.5)	0,498[a]
Urogenital system	6 (12.0)	-	0,318[a]
Genetic/chromosomal abnormalities	5 (10.0)	1 (12.5)	1,000[a]
Cancer	2 (4.0)	-	1,000[a]
Immunosuppressive treatment	5 (10.0)	1 (12.5)	1,000[a]

Table 3 Difference in COVID-19 severity between patients with different comorbid disorders and corresponding p-values

[a] Fisher's exact test [b] Significant value, p < 0,05

Twenty-eight patients were diagnosed with MIS-C as a manifestation of SARS-CoV-2 infection. Twenty-five percent of MIS-C patients (n=7) had a pre-existing comorbidity. Twelve patients (42.9%) met the criteria for critical disease (table 2). The majority of MIS-C patients did not have comorbidities, and no significant associations between comorbidities and severity of MIS-C were found.

The key findings of this study indicate that, although most pediatric patients have non-severe disease, children with pre-existing comorbidities are more likely to have more severe acute COVID-19 than children without comorbidities. In particular, pediatric neurological disorders were associated with more severe COVID-19.

Considerable inconsistency is seen in current evidence on the association between comorbidities and pediatric COVID-19 severity. A meta-analysis by Tsankov et al. combined the findings of several heterogeneous articles, concluding an association between comorbidities and acute COVID-19 severity.[10] However, most of the included studies had a small sample size and only described the association between comorbidities and ICU admission or mortality rate instead of severity as a detailed description of clinical characteristics. This could create selection bias because, children with comorbidities could be admitted to the ICU as a preventative measure instead of due to clinical deterioration.[10] Our study methods included a detailed clinical evaluation to describe COVID-19 disease severity following a classification system in addition to ICU admission.

Corroborating our findings, two studies, performed in other countries and using a similar classification system, found that children with comorbidities have a higher risk for more severe COVID-19, including a larger cohort of 3837 pediatric patients.[11, 12] The other study also found an association between more severe COVID-19 and neurological disorders, which included mostly epilepsy or severe neuro-disability similar to the patients in our study.[11] Another multicenter observational study in the UK also found that among comorbidities in patients that needed critical care due to COVID-19, neurological disorders, such as neuro-disability, were one of the most common.[13] Neurological disabilities such as cerebral palsy, which influence motor functions, could lead to difficulties in spontaneous breathing and clearing respiratory secretions, which could worsen respiratory infections and thus explain this association with more severe acute COVID-19. Moreover, SARS-CoV-2 can affect the nervous system through damage to neuronal cells, muscle tissues and vascular cells, which are likely to be more vulnerable in children with comorbidities.[14]

In contrast, three of the studies that used a similar classification of severity, all with relatively small sample sizes, found that having comorbidities was not associated with disease severity in pediatric patients.[15 - 17] This inconsistency is possibly due to the missing consensus on definitions of relevant pre-existing comorbidities and to a missing universal classification of disease severity of acute COVID-19. In our results we particularly did not find an association between obesity and severity of acute COVID-19, which has been seen in some other studies.[10, 11] It is thought that higher visceral adiposity is associated with higher inflammatory cytokine levels correlated with COVID-19 severity, which might explain why more severe acute COVID-19 can be seen in obese patients.[10] We also do not report an association between respiratory disorders, such as asthma, and acute COVID-19 severity, which is in accordance with findings related to SARS-CoV-1 and MERS-CoV infections.[18] It is suggested that human coronaviruses may not have the capacity to enhance asthmatic inflammation, unlike the human rhinovirus or respiratory syncytial virus.[18]

Our findings imply that having comorbidities is not a risk factor for having more severe MIS-C compared to having no comorbidities. This is in accordance with previous findings that pre-existing comorbidities among MIS-C patients are rare.[3] Healthcare professionals should be aware of the association between pre-existing comorbidities and severity of COVID-19 to determine adequate management strategies for this specific group.
Furthermore, the implications made in this study should be taken into consideration in the debate on SARS-CoV-2 vaccination in children. It is worth noting that the absolute numbers of severe disease due to acute pediatric COVID-19 are low and that the size of the effect comorbidities have on disease severity remains uncertain. However, effects of the COVID-19 pandemic on children in particular, such as social isolation and interruption in education, also should be considered in future management or prevention strategies.[19] This study substantiates the need for large-scale studies with well-defined evaluation and classification of disease severity to determine the true strength of the association.
The strengths of this study include the detailed information on clinical characteristics and outcomes to carefully assess the severity of COVID-19 infection and the association between the severity of MIS-C and pre-existing comorbidities.

Our study has a few limitations. First, the retrospective observational study design may cause residual confounding. Second, because severe COVID-19 and hospital admission is rare in children, our study consisted of a small sample size. Selection bias may have affected the results because asymptomatic or mild children are not always PCR-confirmed and therefore are underrepresented. Owing to the small sample, there was sparsity in numbers included for statistical analyses, which contributes to the broad confidence intervals. Various international databases have been set up to prospectively study COVID-19 severity in patients with comorbidities, a promising development.[10] Third, the study was carried out at a tertiary center where mostly severely ill children or children with complex comorbidities are treated, which can cause selection bias.

In conclusion, our findings show that pediatric acute COVID-19 is mostly non-severe, but children with pre-existing comorbidities are at risk for developing more severe acute COVID-19 compared to patients without comorbidities. MIS-C is generally more severe than acute COVID-19. However, no association was found between comorbidities and severity of MIS-C. More prospective large-scale data on the susceptibility of children with comorbidities for severe acute COVID-19 are needed, as well as more data on risk factors for developing severe MIS-C to establish management strategies for SARS-CoV-2 infections in specific groups of pediatric patients.

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SUPPLEMENTARY MATERIAL

	No. of participants: n = 46
Respiratory disorders	**9 (19.6%)**
Laryngo-/bronchomalacia	3
Asthma	3
Bronchopulmonary dysplasia	1
Primary ciliary dyskinesia (PCD)	1
Subglottic stenosis	1
Lobectomy	1
Systemic auto immune disorders	**3 (6.5%)**
Juvenile idiopathic arthritis (JIA)	2
Kawasaki's disease	1
Neurological disorders	**8 (17.4%)**
Epilepsy/frequent seizures	5
Cerebral palsy	4
Hydrocephalus	2
Cerebral infarction	1
Myasthenia gravis	1
Neuromuscular scoliosis	1
Cardiovascular disorders	**5 (10.9%)**
Congenital cardiac malformation	3
Hypertension	1
Long QT syndrome	2
Re-entry tachycardia	1
Metabolic disorders	**0 (0)**
Endocrine system disorders	**4 (8.7%)**
Diabetes Mellitus (type 1)	1
Thyroid gland disorder	1
Diabetes insipidus	1
Growth hormone deficiency	1
Hematological disorders	**2 (4.3%)**
Sickle cell anemia	2
Gastrointestinal disorders	**3 (6.5%)**
Inflammatory bowel disease (IBD)	1
Esophagus atresia	1
Intestinal perforation	1

Renal/urogenital disorders	**6 (13.0%)**
Bladder or urethral disorders	3
Chronic kidney failure	2
Kidney transplant	3
Kidney dysplasia	1
Duplex collecting system	1
Pyelo-ureteral stenosis	1
Genetic/chromosomal abnormalities	**6 (13.0%)**
Genetic mutations/syndromes	6
Cancer	**2 (4.3%)**
Leukemia	1
Solid tumour	1
Other comorbidities	**3 (6.5%)**
Eating disorder	2
Britt-Hogg-Dube syndrome	1

Supplementary table 1 Detailed description of pre-existing comorbidities

Chapter V

Saliva SARS-CoV-2 antibody prevalence in children

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ABSTRACT

Background

COVID-19 patients produce circulating and mucosal antibodies. In adults, specific saliva antibodies have been detected. Nonetheless, seroprevalence is routinely investigated while little attention has been paid to mucosal antibodies. We therefore assessed SARS-CoV-2-specific antibody prevalence in serum and saliva in children in the Netherlands.

Methods

We assessed SARS-CoV-2 antibody prevalence in serum and saliva of 517 children attending medical services in the Netherlands (irrespective of COVID-19 exposure) between April and October 2020. Prevalence of SARS-CoV-2 spike (S), receptor binding domain (RBD) and nucleocapsid (N)-specific IgG and IgA were evaluated with an explorative Luminex assay in serum and saliva and with the Wantai SARS-CoV-2 RBD total antibody enzyme-linked immunosorbent assay in serum.

Results

Using the Wantai assay, the RBD-specific antibody prevalence in serum was 3.3% (95% CI 1.9 – 5.3%). With the Luminex assay we detected heterogeneity between antibodies for S, RBD and N antigens, as IgG and IgA prevalence ranged between 3.6% – 4.6% in serum and between 0% – 4.4% in saliva. The Luminex assay also revealed differences between serum and saliva, with SARS-CoV-2-specific IgG present in saliva but not in serum for 1.5 – 2.7% of all children. Using multiple antigen assays, the IgG prevalence for at least two out of three antigens (S, RBD or N) in serum or saliva can be calculated as 3.8% (95% CI 2.3 – 5.6%).

Conclusions

Our study displays the heterogeneity of the SARS-CoV-2 antibody response in children and emphasizes the additional value of saliva antibody detection and the combined use of different antigens.

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a positive-sense single-stranded RNA virus from the Coronaviridae family causing coronavirus disease 2019 (COVID-19). In 2020, this novel virus emerged as the cause of a pandemic. In the Netherlands, the first COVID-19 patient was confirmed on February 27th 2020. After the first peak in hospital admission rates during March and April 2020, the Netherlands endured a second peak during the second half of 2020. Epidemiological data on immunity is essential to understand disease pathology, to guide national prevention measures and possibilities for vaccine development.[1]

Generally, humoral immunity is measured as the presence of pathogen-specific antibodies in serum. Prevalence of SARS-CoV-2 antibodies in serum has been described in several countries including the Netherlands,[2-4] with a potential durability of over 8 months.[5, 6] Although the mucosa of the upper respiratory tract is the primary entry site of SARS-CoV-2, little attention has been paid to the presence of mucosal antibodies as part of the humoral immune response.[7] For other viruses such as Hepatitis B virus, Norovirus and human immunodeficiency virus type 1, studies have shown high similarity in circulating and mucosal antibody profiles, advocating for the use of saliva samples to measure humoral immunity.[8-10] In respiratory syncytial virus (RSV), mucosal anti-RSV IgA and IgG combined proved at least as reliable as serum to detect infection. These saliva antibodies could help to distinguish current RSV (re-)infection from a false-positive result due to pre-existing maternal antibodies.[11] In adult COVID-19 patients, promising results on SARS-CoV-2 specific saliva antibodies with neutralizing capacities and a durability similar to serum have been reported.[12-14] Interestingly, in asymptomatic or mild COVID-19, mucosal antibodies were detected, even in some seronegative patients.[15] Mucosal antibody measurement could be an important and more convenient tool to evaluate humoral immunity in children, since they often have asymptomatic or mild disease.[16, 17] Among children, circulating antibody prevalence ranges from 0.9% in the United States to 7.3% in Switzerland.[16, 18] The use of saliva antibody assays has yet to be explored in asymptomatic cases and in a pediatric population. Thus, the primary aim of this study was to evaluate the SARS-CoV-2-specific antibody prevalence in saliva compared to serum in a pediatric population during the COVID-19 outbreak in the Netherlands. Since the Luminex assay provides a very sensitive method which is easily adjustable to different antigens, antibody isotypes and sample types, we have utilized this explorative assay in addition to the validated Wantai.

METHODS

Study design and participants

This prospective cross-sectional study included simultaneous convenience blood and saliva sampling of children attending medical services at one of seven participating secondary and tertiary care hospitals in the North-West region of the Netherlands during 24 consecutive weeks (April 12th to October 2nd 2020). Inclusion criteria were children aged 0 to 18 years residing in the Netherlands, who required blood testing or intravenous cannulation for any reason. Eligibility was irrespective of (suspected) acute or prior COVID-19 infection. Children were excluded if sample collection of neither serum nor saliva was sufficient.

We recorded age, sex, COVID-19 related symptoms and proven or suspected COVID-19 in household members. COVID-19 related symptoms were defined as: fever (> 37.5 °C), sore throat, cough, shortness of breath, tachypnea, headache, abdominal cramps, diarrhea. Electronic patient files were used to extract previous SARS-CoV-2 polymerase chain reaction (PCR) assay results and medical history that could influence the humoral immune response or infection severity. Children with immunodeficiency, autoimmune disease, hematological malignancies and/or use of immunomodulating drugs were defined as having an 'immunocompromised state'. Immunomodulating drugs included: azathioprine, methotrexate, monoclonal antibodies, immunoglobulins and corticosteroids. We defined children with an 'underlying illnesses' as children with obesity (BMI ≥ 30), respiratory, cardiovascular, endocrine, metabolic, hematologic, or kidney diseases, solid malignancies, or psychomotor retardation. Previously healthy children were categorized as having 'no relevant medical history'. The study protocol was approved by the ethics committee of the Amsterdam University Medical Centers (NL73556.018.20) and conducted in accordance with good clinical practice standards. Written informed consent was obtained from both parents/guardians and/or from children above the legal age of consent.

Serum and saliva sampling

Saliva was obtained using a sterile container or a buccal swab (ORACOL Saliva Collection Device, Product Code S10, Malvern Medical Developments Ltd). Saliva samples were either stored at -70° C until centrifuging or directly centrifuged (at 1000 rpm for 10 minutes). Supernatant and pellets were stored in aliquots at -80° C. During venipuncture a blood sample of 1 to 5 ml was collected, centrifuged and serum stored at -20° C.

Serum assays

Seroprevalence was assessed with the FDA approved Wantai SARS-CoV-2 Receptor Binding Domain (RBD) total antibody enzyme-linked immunosorbent assay following manufacturer's instructions, with a sensitivity of 96.7% (95% confidence interval [CI] 83.3% - 99.4%) and specificity of 97.5% (95% CI 91.3% - 99.3%),[19] and confirmed with an in-house developed SARS-CoV-2 RBD total antibody bridge assay as described previously (sensitivity 98.1% and specificity 99.5%).[20]

Protein coupling to Luminex beads

An explorative Luminex assay was developed to investigate antigen-specific IgG and IgA in serum and saliva. A recombinant prefusion ectodomain trimer of SARS-CoV-2 spike (S) protein, the monomeric RBD of the S protein and the nucleocapsid (N) protein were designed, produced and purified as previously described.[21, 22] The proteins were covalently coupled to Magplex beads (Luminex) using a two-step carbodiimide reaction at a ratio of 75 µg protein to 12.5 million beads for S, at equimolar concentration for N and at 3x the equimolar concentration for RBD. Beads were washed with 100 mM monobasic sodium phosphate pH 6.2, activated with Sulfo-N-Hydroxy-sulfosuccinimide and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (Thermo Fisher Scientific) and incubated for 30 minutes on a rotator at room temperature (RT). Activated beads were washed 3x with 50 mM MES pH 5.0, proteins were added, and beads were incubated for three hours on a rotator at RT. The beads were washed with PBS and blocked with PBS containing 2% BSA, 3% Fetal calf serum and 0.02% Tween-20 at pH 7.0 (PBS-blocking) for 30 minutes on a rotator at RT. Finally, the beads were washed, stored in PBS containing 0.05% Sodium Azide at 4°C and used within six weeks.

Luminex assays

50 µl of PBS-blocking containing 20 of each bead per µl was incubated with 50 µl of 1:10,000 diluted serum or 1:10 diluted saliva supernatant in PBS-blocking overnight on a plate shaker at 4°C. Subsequently, plates were washed with TBS containing 0.05% Tween-20 (TBST) using a magnetic separator. Beads were resuspended in 50 µl of Goat-anti-human IgG-PE (Southern Biotech) or Goat-anti-human IgA-PE (Southern Biotech) in PBS-blocking and incubated on a plate shaker at RT for two hours. Afterwards, beads were washed with TBST and resuspended in 70 µl Magpix drive fluid (Luminex). Read-out was performed on a Magpix (Luminex). Resulting median fluorescence intensity (MFI) values per bead were background-corrected by subtraction of the MFI values of buffer only. A titration of serum and saliva of an adult convalescent COVID-19 patient was used to normalize data between plates. The cut-off was determined at 2 (geometric) standard-deviations above the geometric mean of the total sample (n = 509 for serum, n = 430 for saliva), separately for each combination of antigen, sample type and secondary antibody (supplementary table 1). Beads with no antigen were included to confirm absence of antibodies binding to beads or blocking components for each individual sample. Pre-pandemic serum pools (n=3) or healthy donor saliva (early in pandemic with negative PCR and/or absence of symptoms; n = 4) were included on each assay plate as negative controls. The replicability was calculated on samples measured twice and the assay precision was calculated using positive control sera (n=6) or saliva (n = 5) included on each plate (supplementary table 1).

Statistical analyses

We estimated prevalence of circulating and mucosal antibodies as the proportion of children with a result above the cut-off value. Confidence intervals were calculated with the Clopper-Pearson method in IBM SPSS Statistics (Version 26) predictive analytics software. In accordance with the World Health Organization's Investigation Protocol for COVID-19 Seroepidemiological research, we stratified participants into pre-defined age groups: < 1

year, 1-4 years, 5-9 years, 10-14 years and 15-17 years.[1] We calculated a minimum sample size of 139 children per age group (0-1 year, 1-5, 5-18 years) to detect a seroprevalence of 10% (95% CI 5-15%).

RESULTS

Study population

A total of 589 children were approached, of which 517 children were included (supplementary figure 1). Characteristics of participants are shown in supplementary table 2. Figure 1a shows the age distribution across the inclusion period. The median age was 11 years (IQR 5 – 15 years). An immunocompromised state and an underlying illness were described in 35.8% and 24.8% of children respectively, while 38.9% did not have a relevant medical history. Sex was equally distributed among comorbidity groups (figure 1b). SARS-CoV-2 PCR on nasopharyngeal swabs had been performed previously by the treating physician in 107 children, either due to clinical suspicion of COVID-19 or as pre-procedural testing. Paired serum and saliva samples of sufficient volume for all assays were available for 413/517 (82%) children, with a median age of 12 years (IQR 7 – 15 years).

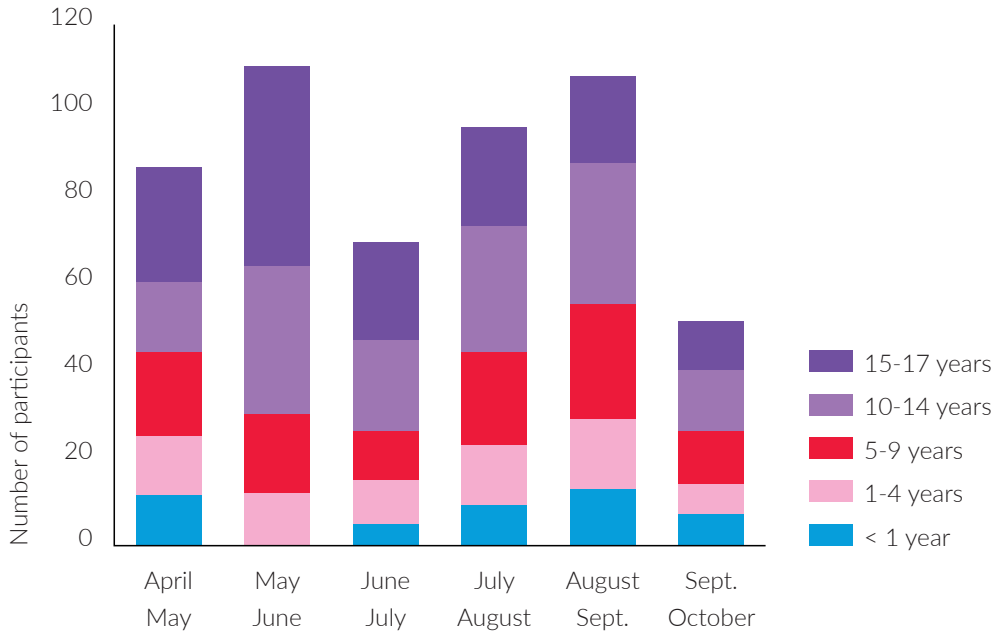

Figure 1a The distribution of age across the inclusion period of 24 weeks is depicted for the total of cases (n=517) with each bar representing four weeks.

Antibody prevalence

Figure 2 shows the estimated prevalence determined by the Wantai RBD total antibody assay (a) and the Luminex assay for S, RBD and N-specific IgG (b) and IgA (c) in serum and saliva. Titers from the Luminex assay are shown in supplementary figure 2 and the assay cut-off and assay performance parameters in supplementary table 1. Prevalence of RBD-specific antibodies in serum was 16/487 (3.3%, 95% CI 1.9 – 5.3%) with the Wantai assay. With the Luminex assay, prevalence of SARS-CoV-2-specific antibodies in serum ranged between 3.3% (95% CI 1.9 – 5.2%) and 4.3% (95% CI 2.7 - 6.4%) depending on the antigen and isotype measured. The prevalence of SARS-CoV-2-specific antibodies in saliva ranged between 0.0% (95% CI 0.0 – 0.7%) and 4.4% (95% CI 2.7 - 6.8%).

Prevalence of SARS-CoV-2-specific antibodies did not increase over calendar months in the Luminex assays nor the Wantai assay (supplementary figure 3). Seroprevalence was 4/200 (2.0%) in children aged 0 to 10 years and 12/287 (4.2%) in children aged 10 to 17 years in the Wantai assay (supplementary table 2). Seroprevalence was 3.3% in children with an immunocompromised state, 5.0% in children with underlying illness and 2.2% in children with no relevant medical history. Most of the children that were positive in the Wantai assay (13/16, 81%) had COVID-19 symptoms at time of inclusion, or in the previous four weeks or reported a COVID-19 positive household member.

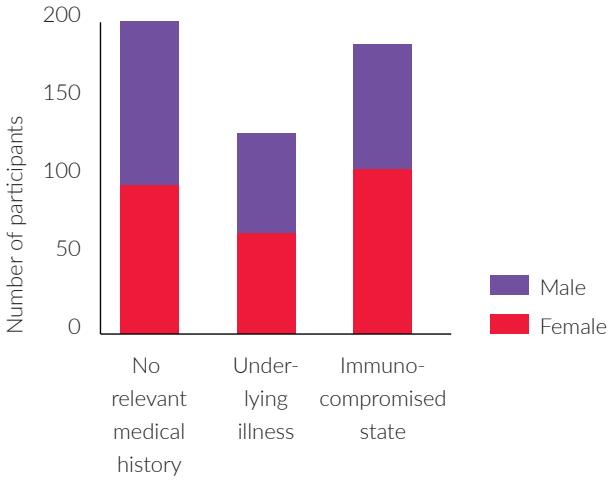

Figure 1b Comorbidity and male/female ratio of the study sample is calculated for the non-missing values (n=514, excluding three missing values for comorbidity).

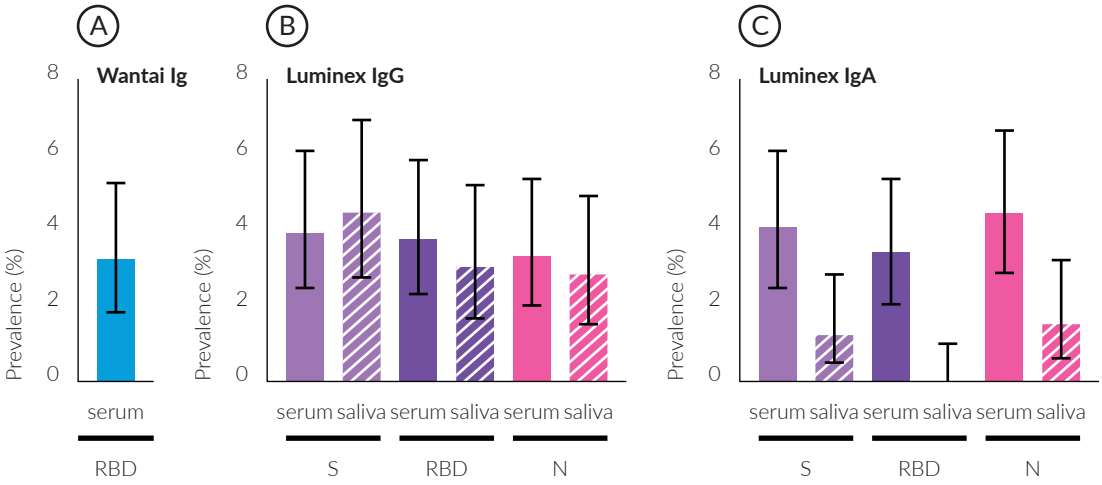

Figure 2 SARS-CoV-2 antibody prevalence estimates in serum and saliva
(a) Prevalence estimate of SARS-CoV-2 RBD total antibodies in the Wantai assay for serum. (b, c) Prevalence estimates of SARS-CoV-2 S, RBD and N specific IgG (b) and IgA (c) in the Luminex assays for serum [solid bars] and saliva [hatched bars]. Prevalence was the calculated proportion with a value above the determined cut-off out of non-missing values. Estimates are shown with 95% confidence intervals. Abbreviations: S = trimeric SARS-CoV-2 spike protein, RBD = the monomeric receptor binding domain of the SARS-CoV-2 spike protein, N = SARS-CoV-2 nucleocapsid protein.

COMPARISON OF SERUM AND SALIVA SARS-COV-2 ANTIBODY PREVALENCE

In the Luminex assay, 31/422 (7.4%) children had detectable S-specific IgG, while 22/422 (5.2%) had detectable RBD-specific IgG and 21/422 (5.0%) had detectable N-specific IgG in serum and/or saliva (figure 2b). Figure 3a shows the correspondence between serum and saliva IgG for all paired samples in the Luminex and Wantai assays. Between 7/31 and 6/21 (23 – 29%) of these children showed corresponding positive titers in both serum and saliva, depending on the antigen used. However, 12/31 – 9/21 (38 – 43%) children had positive SARS-CoV-2-specifc IgG titers in serum but not in saliva and 6/21 – 12/31 (29 – 39%) had positive titers in saliva but not in serum.

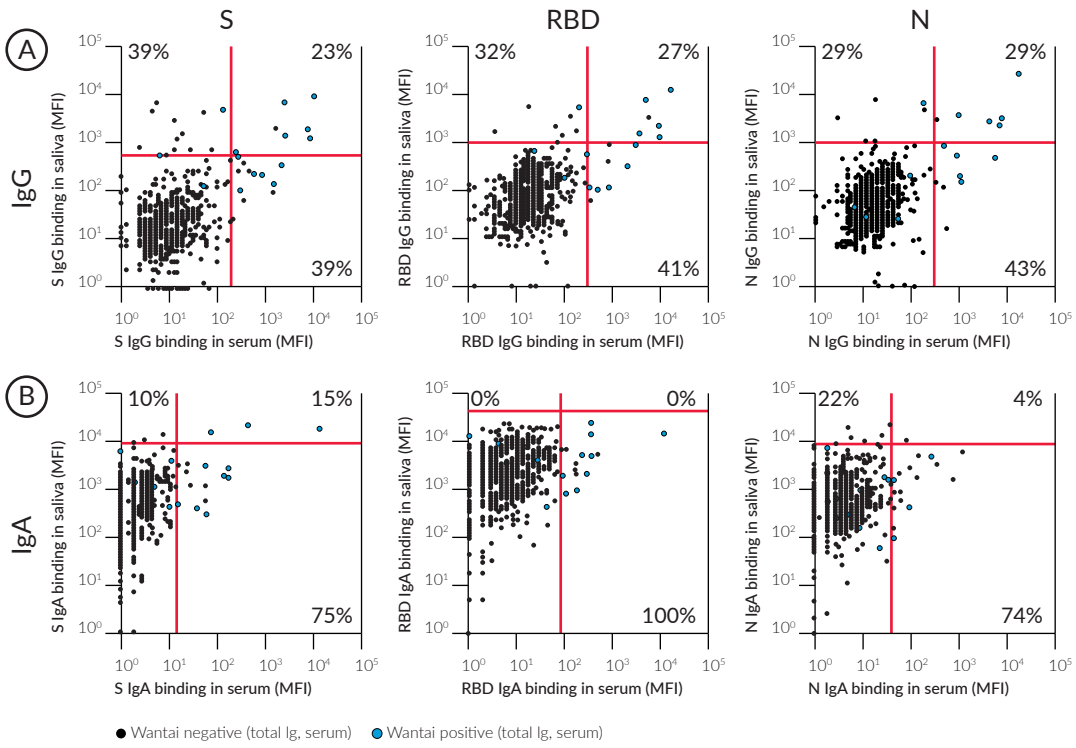

Figure 3 Correspondence of Luminex assays for serum and saliva

SARS-CoV-2 S, RBD and N-specific IgG (a) and IgA (b), measured in paired serum and saliva samples (n=413) by Luminex assay, expressed as MFI. Only samples also measured in the Wantai assay are shown with the positive Wantai results indicated in blue. Serum and saliva are plotted against each other to reveal the differences between each compartment. The red dotted lines are the cut-off values to discriminate positive and negative measurements in the Luminex assays. Percentages represent data points which are positive for both compartments or for a single compartment as the percentage of total positives in each graph. Abbreviations: S = trimeric SARS-CoV-2 spike protein, RBD = protein of only the monomeric receptor binding domain of the SARS-CoV-2 spike protein, N = SARS-CoV-2 nucleocapsid protein, MFI = median fluorescence intensity.

Comparable with these findings, between 6/15 – 8/15 (40 – 53%) of Wantai positive children also had positive saliva IgG titers in the Luminex, but between 6/12 and 11/19 (50 - 58%) of children with SARS-CoV-2-specific saliva IgG in the Luminex were negative in the Wantai. This corresponds to 6 – 11/413 (1.5 – 2.7%) of the total paired sample, depending on the antigen used.

We compared IgA responses in serum and saliva (figure 3b), but there was low agreement between the two compartments (0/15 – 3/20; 0 – 15% with detectable IgA in both serum and saliva). The Luminex IgA assays were positive in saliva while negative in serum in 0/15 – 5 / 23 (0 – 22%) of children with IgA antibodies.

COMBINED LUMINEX ASSAY

Figure 4a shows the correspondence between the three SARS-CoV-2-specific antigens. All children with S and N-specific IgG in serum also had positive titers of RBD-specific IgG. Comparing IgG and IgA antibodies (figure 4b) in serum, a few children showed S-specific IgA but not IgG antibodies (10/509; 2.0%). All but one child positive for both serum IgG and IgA in the Luminex assay were also positive in the Wantai total antibody assay (9/487; 1.8%). There was low correspondence between IgG and IgA in saliva.

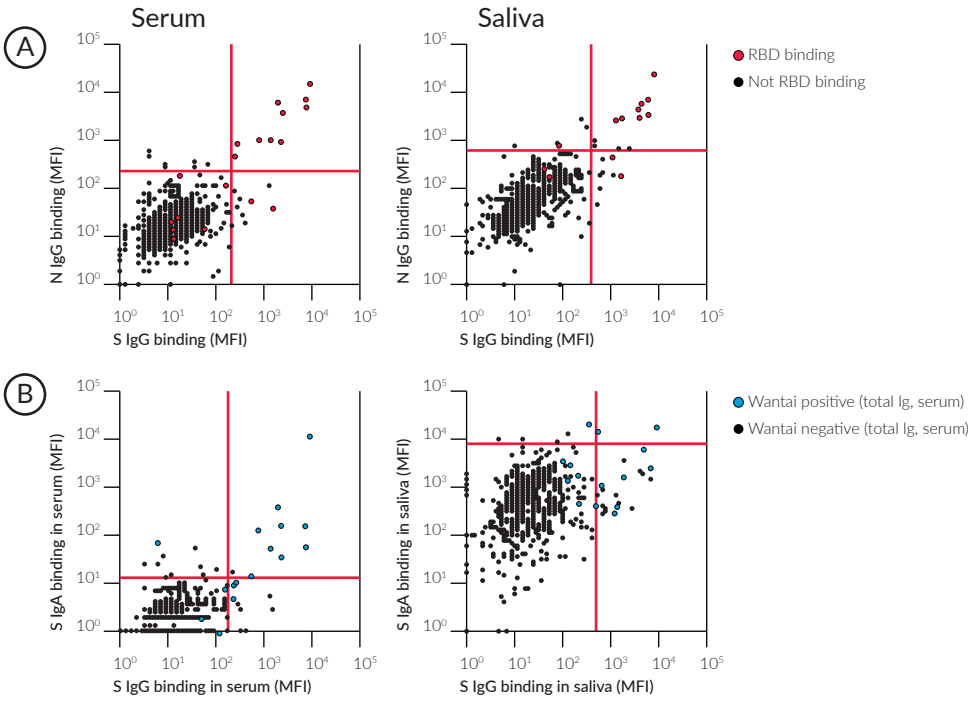

Figure 4 Correspondence of Luminex assays for different antigens and isotypes
(a) S, RBD and N protein specific IgG, measured in serum (n=509, left) and saliva (n=430, right) in the Luminex assay, expressed as MFI. S and N are plotted against each other and RBD positive samples are shown in orange. The red lines are the cut-off values to discriminate positive and negative measurements for S and N. (b) SARS-CoV-2 S-specific IgG and IgA in serum (n=487, left) and saliva (n=413, right) measured in the Luminex assay, expressed as MFI. IgG and IgA are plotted against each other to reveal the correspondence between the two isotypes. Samples that were also positive in the Wantai RBD total antibody assay are shown in blue. Abbreviations: S = trimeric SARS-CoV-2 spike protein, RBD = monomeric receptor binding domain of the SARS-CoV-2 spike, N = SARS-CoV-2 nucleocapsid protein, MFI = median fluorescence intensity.

We have explored combining multi-antigen assays to calculate the SARS-CoV-2 IgG prevalence in several ways (figure 5). Total prevalence of IgG binding to any of the antigens is 34/509 (6.7%) in serum and 26/430 (6.0%) in saliva. To increase the specificity, the IgG prevalence for at least two out of three antigens (S, RBD or N protein) can be calculated as 2.4% for serum, 2.3% for saliva and 3.8% (95% CI 2.3 – 5.6%) when both serum and saliva are measured. Considering only children with positive Luminex titers for two out of three antigens in saliva, 4/413 children had saliva antibodies while they were negative in the Wantai assay, corresponding to 1.0% (95% CI 0.3 - 2.2%) of the total sample.

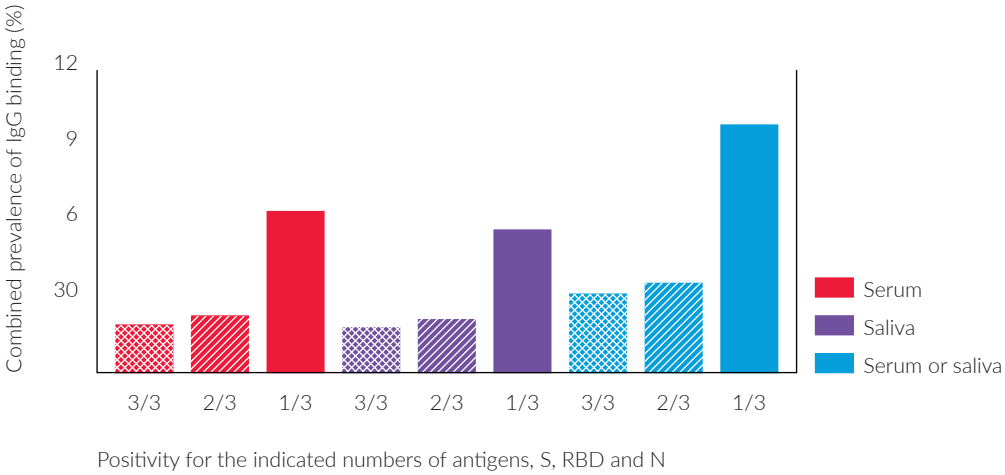

Figure 5 Combined SARS-CoV-2 IgG antibody prevalence
Combined prevalence of the explorative Luminex assay of IgG in serum [red bars], saliva [purple bars] or in either serum or saliva combined [blue bars]. The combined prevalence was calculated for children positive for 3/3 SARS-CoV-2 antigens [crosshatched bars] at least 2/3 antigens [hatched bars] and for children positive for at least 1/3 antigen [solid bars]. Abbreviations: S = trimeric SARS-CoV-2 spike protein, RBD = monomeric receptor binding domain of the SARS-CoV-2 spike, N = SARS-CoV-2 nucleocapsid protein.

DISCUSSION

This is the first study evaluating concurrent mucosal and circulating SARS-CoV-2-specific antibodies in a large pediatric cohort. We found children with detectable SARS-CoV-2-specific saliva IgG while circulating antibody levels (measured with the Wantai) were undetectable. Similarly, not all seropositive children had detectable saliva IgG. Additionally, we detected heterogeneity in the presence of antibodies to different SARS-CoV-2 specific antigens.

We found an antibody seroprevalence comparable to the S-specific IgG seroprevalence among 1000 Dutch children during June and July 2020 in a population-based study.[23, 24] Most children in our population had an underlying illness or immunocompromised state. Therefore, the similarities to national seroprevalence studies are particularly interesting and suggest similar COVID-19 incidence in our hospital-based cohort compared to the general community. Other European countries also report lower seroprevalence in children (range 0.8 – 7.3%) compared to adults (range 1 – 20%).[18, 25]

Although many reports are appearing on SARS-CoV-2 seroprevalence, the initial confrontation of SARS-CoV-2 with the adaptive immune system is located at the mucosal surface of the respiratory tract. To our knowledge, merely a few adult cohorts report on the associations between circulating and mucosal SARS-CoV-2-specific an-

tibodies. For example, a sensitivity of 84.2% and a specificity of 100% for saliva S-specific IgG to detect PCR- and seropositive patients in a symptomatic population was reported.[12] The durability appears similar to circulating antibodies, as salivary antibodies were shown to be measurable for up to nine months after infection in convalescent mild COVID-19 patients.[26] Three cohorts of SARS-CoV-2 infected adults suggest that mucosal IgG antibodies can be used as a surrogate for circulating IgG due to the high similarity.[12-14]
However, a proportion of seronegative children in our sample did have saliva antibodies (mostly IgG). Corroborating these findings, 15-20% of seronegative health care workers had mucosal SARS-CoV-2 S-specific antibodies with, in some cases, comparable in-vitro neutralizing capacity to serum.[15, 27] A possible explanation for the discordance between circulating and mucosal antibodies could be the disease severity. Cervia et al. hypothesize that the mucosal antibody response is more prominent in mild infection and in younger individuals, although their youngest participant was 30 years.[15] Similarly, a preprint validation study of a saliva IgA assay observed asymptomatic individuals with saliva SARS-CoV-2-specific antibodies despite a negative PCR and/or serum antibody tests.[28] Hence, reporting seroprevalence alone may result in an underestimation of humoral immunity, particularly in younger and mildly infected patients.

We still lack full understanding on how IgA provides additional value for evaluating humoral immunity.[29] IgA can appear earlier in blood than IgG following SARS-CoV-2 infection.[30] Although IgA is the key immunoglobulin for mucosal immunity, evidence on SARS-CoV-2-specific saliva IgA is inconsistent. Compared to IgG, saliva IgA is less correlated with serum IgA.[3] Saliva IgG is mostly derived from circulatory IgG through transudation whereas saliva IgA can be produced locally.[31] This is also observed in vaccination response studies, showing saliva S-specific IgG in all fully vaccinated participants while only 60% showed saliva S-specific IgA.[32] Consistent with our findings, MacMullan and Pisanic emphasize the lower sensitivity for IgA to detect PCR-positive patients as compared to IgG.[12, 13] The high variation in mucosal IgA titers complicates detection of SARS-CoV-2-specific saliva IgA, possibly caused by polyreactive IgA known to function as a non-specific mucosal immune barrier.[28]

There is growing evidence on the use of SARS-CoV-2 multi-antigen assays in epidemiological surveys. In line with other studies, we observed that positive individuals often do not show equally elevated titers across all three SARS-CoV-2 specific antigens. In an epidemiological survey of 1225 blood donors, seroprevalence with single-antigen assays also varied widely between 0.8% and 7.5% depending on the antigen type.[33] A potential association between specific antigens and disease severity has been proposed. Outpatients with mild or asymptomatic infection showed higher ratios of S and RBD-specific antibodies compared to N-specific antibodies, whereas all three antigens were effective for detecting responses in hospitalized patients.[27, 34] Considering their different kinetics[35] and functions in the humoral immune response and the broad clinical spectrum of COVID-19, combining multiple SARS-CoV-2 antigens seems an appropriate method when investigating population prevalence.[36]

Targeting multiple antigens in multiple compartments to evaluate the humoral response can increase sensitivity, but may consequently increase false-positives. A golden standard is unfortunately still missing to determine true rates, as both PCR and serology can have false-negative results.[37, 38] To increase certainty of SARS-CoV-2 exposure and minimize the possibility of a false-positive (particularly in a presumed low prevalence context),[39] we could define a sample as positive only when the IgG level is above the cut-off for at least two antigen assays as proposed in other studies.[13, 35, 36, 40] In adult cohorts of (symptomatic) PCR-proven COVID-19 patients and pre-COVID-19 era controls, combining multiple N and S/RBD antibody assays resulted in higher diagnostic accuracy.[13, 36, 40, 41] Although Luminex assays in serum are known to have high accuracy to detect previous SARS-CoV-2 infection[35, 36, 40, 41] and the validated Wantai assay provides context for the results, follow-up research should focus on validation of established assays on materials other than serum as well as the combined use of different antigens in high and low prevalence settings.

Our study encountered several other limitations. We did not evaluate the potential moment of infection, thus we could have missed SARS-CoV-2 exposed children as IgG can be detected after 10 days and for several months

post symptom-onset while IgM and IgA wane more quickly.[3] We minimized this effect by detecting multi-isotype antibodies. Moreover, cross-reactive antibodies from previous coronavirus infections could have resulted in positivity without a history of actual SARS-CoV-2 infection. Antibody cross-reactivity is a known phenomenon for pathogens with shared structural motifs.[42, 43] In a cohort sampled in the pre-COVID-19 era, detectable SARS-CoV-2 S-specific antibodies were found and at higher frequency in children as compared to adults, peaking to 62% between 6-16 years of age.[43] Although these antibody titers were lower than in COVID-19 patients, their sera exhibited neutralizing activity against SARS-CoV-2.[43] Thus, even if some of the antibody titers we found are the result of cross-reactivity, they could still be functional. Since we did not perform in-vitro neutralization testing, the functionality of the (mucosal) antibodies in our cohort against SARS-CoV-2 remains unknown.

CONCLUSION AND IMPLICATIONS

Comprehending humoral immunity to SARS-CoV-2, including in children, is crucial for future public health and vaccine strategies. We therefore detected SARS-CoV-2 antibody prevalence in serum and saliva of children. Our study displays the heterogeneity of the SARS-CoV-2 antibody response in children and emphasizes the additional value of saliva assays for antibody detection as well as the combined use of different antigens. Validation of multi-antigen saliva antibody assays is recommended for further research.

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SUPPLEMENTARY MATERIAL

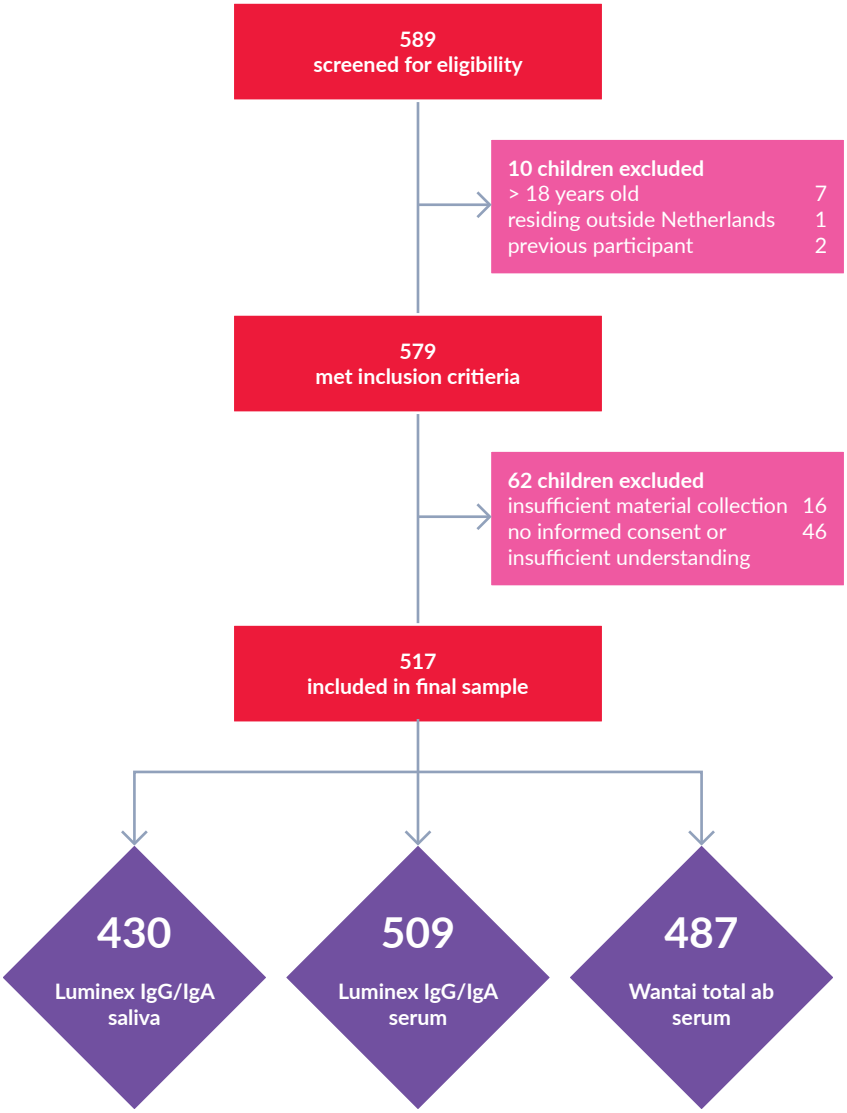
	Serum IgG			Serum IgA			Saliva IgG			Saliva IgA		
	S	RBD	N	S	RBD	N	S	RBD	N	S	RBD	N
Mean	0.30	0.59	0.99	0.54	1.52	0.93	0.90	0.82	0.75	2.40	2.60	2.75
SD	0.49	0.58	0.59	0.59	0.71	0.81	0.62	0.61	0.58	0.71	0.97	1.03
Cut-off	1.27	1.74	2.16	1.71	2.93	2.54	2.15	2.04	1.91	3.81	4.54	4.80
R^2	0.89	0.89	0.90	0.94	0.79	0.67	0.68	0.69	0.73	0.94	0.83	0.91
CV	1-4%	1-4%	1-4%	2-5%	2-5%	1-5%	2-8%	4-7%	4-9%	2-3%	2-5%	3-5%

Supplementary table 1 Mean, standard deviation, assay cut-off, replicability and precision of Luminex assays
Mean, standard deviation (SD) and assay cut-off (mean + 2x SD) are calculated and presented as log10 values. R^2 presents the data replicability and is calculated by simple linear regression on the data in this study plotted against a replicate measurement of data using the same assay conditions on a different day. CV = coefficient of variation, the CV was calculated for each positive control sample and the range is shown.

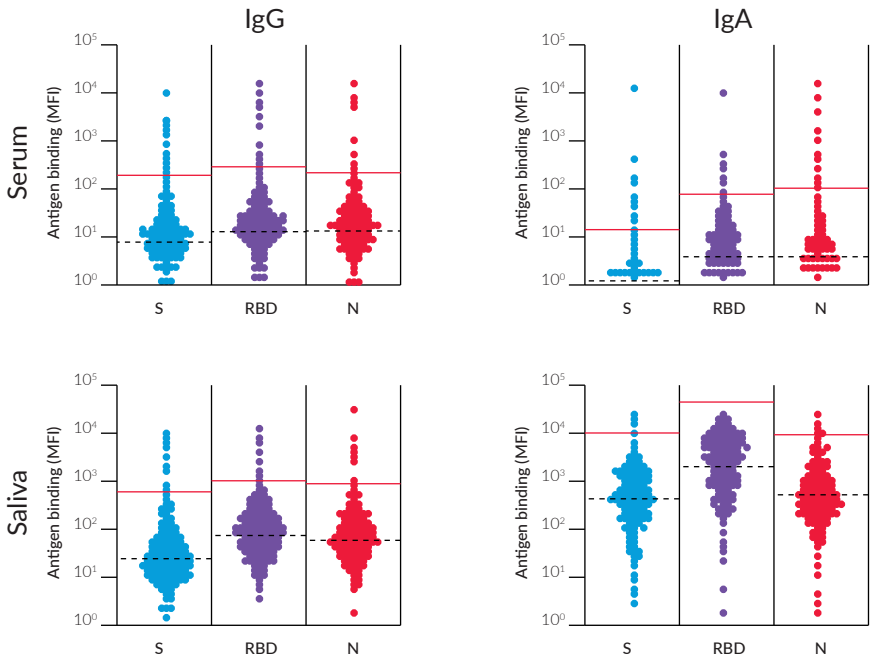
	Total sample (percentage)	Seronegative Wantai No (percentage)	Seropositive Wantai No (percentage)
Total sample	517 (100%)	471 (96.7%)	16 (3.3%)
Sex			
Female	265 (51.3%)	242 (96.0%)	10 (4.0%)
Male	252 (48.7%)	229 (97.4%)	6 (2.6%)
Age (years)			
< 1	47 (9.1%)	34 (100.0%)	0 (0.0%)
1-4	71 (13.7%)	63 (98.4%)	1 (1.6%)
5-9	107 (20.7%)	99 (97.1%)	3 (2.9%)
10-14	146 (28.2%)	139 (95.9%)	6 (4.1%)
15-17	146 (28.2%)	136 (95.8%)	6 (4.2%)
Inclusion			
April/May	86 (16.6%)	75 (94.9%)	4 (5.1%)
May/June	109 (21.1%)	100 (97.1%)	3 (2.9%)
June/July	69 (13.3%)	64 (95.5%	3 (4.5%)
July/August	95 (18.4%)	88 (97.8%)	2 (2.2%)
August/September	107 (20.7%)	97 (96.0%)	4 (4.0%)
September/October	51 (9.9%)	47 (100.0%)	0 (0.0%)
Immunocompromised state	**185 (35.8%)**	**176 (96.7%)**	**6 (3.3%)**
Immunodeficiency	2.9%		
Autoimmune disease	30.8%		
Hematological malignancies	0.8%		
Use of immunomodulating drugs	34.7%		

	Total sample (percentage)	Seronegative Wantai No (percentage)	Seropositive Wantai No (percentage)
Underlying illness	128 (24.8%)	114 (95.0%)	6 (5.0%)
- Obesity	2.9%		
- Respiratory	4.8%		
- Cardiovascular	6.2%		
- Diabetic	0.4%		
- Other malignancies	1.9%		
- Endocrine/metabolic	5.4%		
- Kidney disease	2.3%		
- Hematologic	2.1%		
- Psychomotor retardation	0.8%		
No relevant medical history	201 (38.9%)	180 (97.8%)	4 (2.2%)
Unknown	3 (0.6%)	3 (100.0%)	0 (0.0%)
Type of hospital visit			
- Day-care	271 (52.4%)	244 (94.9%)	13 (5.1%)
- Outpatient	103 (19.9%)	100 (99.0%)	1 (1.0%)
- ER visit	27 (5.2%)	27 (100.0%)	0 (0%)
- Inpatient	110 (21.3%)	94 (97.9%)	2 (2.1%)
- Unknown	6 (1.2%)	36 (100.0%)	0 (0%)
Type of hospital			
- Tertiary care center	363 (70.2%)	327 (95.3%)	16 (4.7%)
- Secondary care center	154 (29.8%)	144 (100%)	0 (0%)
COVID-19 symptoms at time of inclusion			
- Yes	138 (26.7%)	130 (98.5%)	2 (1.5%)
- No	377 (73.0%)	339 (96.0%)	14 (4.0%)
COVID-19 symptoms in last 4 weeks			
- Yes	232 (44.9%)	210 (94.6%)	12 (5.4%)
- No	280 (54.2%)	257 (98.5%)	4 (1.5%)
COVID-19 in household			
- Yes	10 (1.9%)	5 (50.0%)	5 (50.0%)
- No	497 (96.1%)	456 (97.6%)	11 (2.4%)
Prev. SARS-CoV-2 PCR Positive	3/107 (2.8%)	1 (33.3%)	2 (66.7%)

Supplementary table 2 Study population characteristics and seroprevalence
Characteristics are described for all children included in at least one of the assays (n = 517). Prevalence was calculated as number (percentage) from non-missing values for either the Wantai RBD total antibody assay (n=487, 30 missing) or the independent variable. Abbreviations: COVID-19 = coronavirus disease 2019, ER = emergency room, No = number, PCR = polymerase chain reaction assay, RBD = Receptor binding domain, SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2.

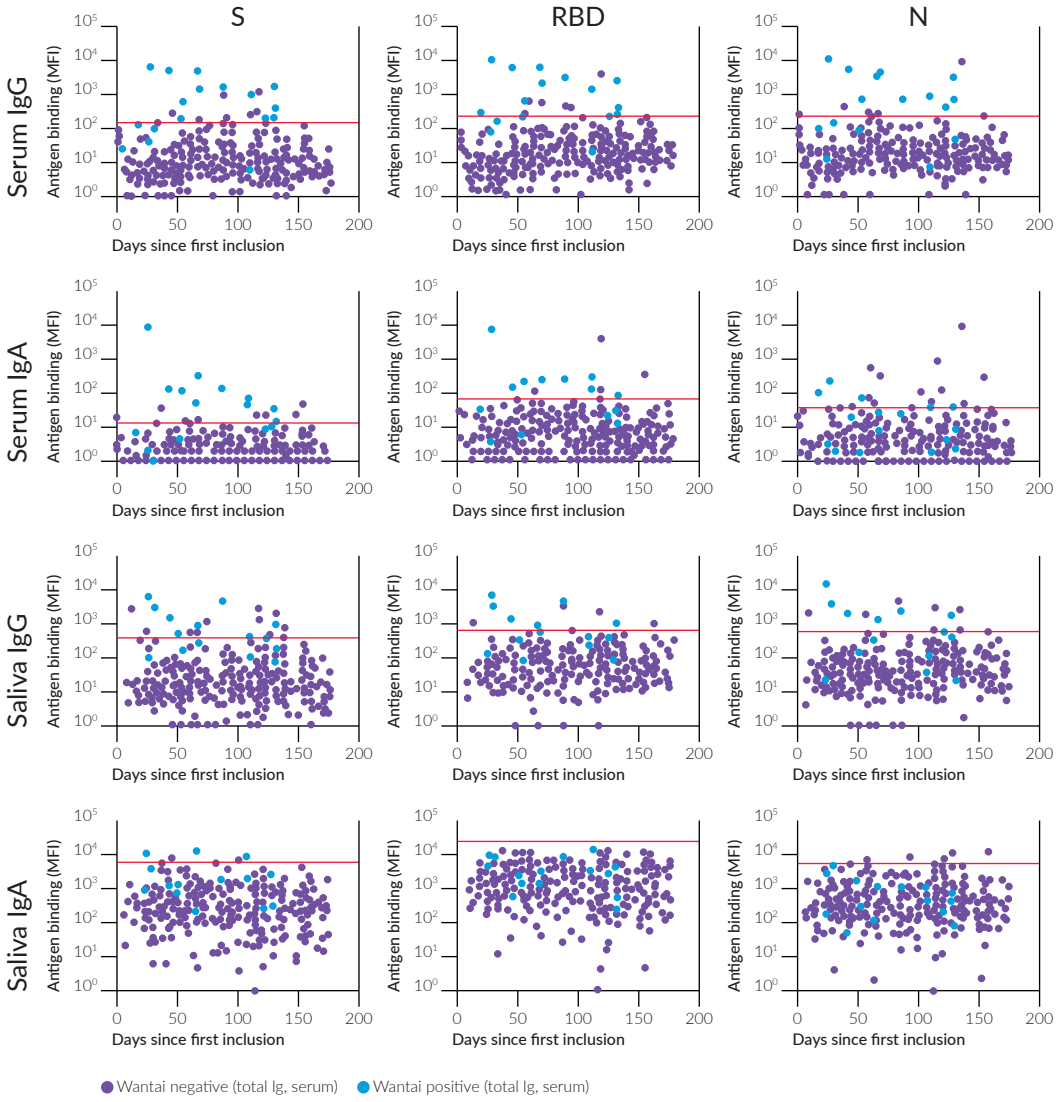


Supplementary figure 1 Participant recruitment and inclusion
The inclusion process is depicted for the total study sample (n=517). Differences in number of serum samples analyzed between the Luminex and the Wantai RBD total antibody assays are due to the serum volumes required for the Luminex (2,5 µl per sample) and the Wantai (200 µl per sample). Differences between total study sample and number of saliva samples are due to insufficient sampling.



Supplementary figure 2 IgG and IgA Titers in the Luminex assay

SARS-CoV-2 S, RBD and N protein specific IgG and IgA were measured in all serum (n=509) and saliva (n=430) samples with the Luminex assay, expressed in MFI. The cut-off for a positive result was determined using the geometric mean + 2x the geometric standard deviation, for each combination of sample type, antibody isotype and antigen separately (see supplementary table 1). All dots represent individual data points, the dashed black line is the geometric mean and the red the cut-off. Abbreviations: S = trimeric SARS-CoV-2 spike protein, RBD = the monomeric receptor binding domain of the SARS-CoV-2 spike protein, N = SARS-CoV-2 nucleocapsid protein, MFI = median fluorescence intensity.



Supplementary figure 3 Serum and saliva antibody prevalence over time

SARS-CoV-2 S, RBD and N-specific IgG and IgA were measured in serum (n=487) and saliva (n=413) samples with the Luminex assay, expressed as MFI. Only samples also measured in the Wantai assay are shown with the positive Wantai results indicated in blue. The cut-off for a positive result in the Luminex was determined using the geometric mean + 2x the geometric standard deviation, for each combination of sample type, antibody isotype and antigen separately (see supplementary table 1). All dots represent individual data points, and the red line is the assay cut-off. Data is plotted against the days since first inclusion. Abbreviations: S = trimeric SARS-CoV-2 spike protein, RBD = the monomeric receptor binding domain of the SARS-CoV-2 spike, N = SARS-CoV-2 nucleocapsid protein, MFI = median fluorescence intensity.

Chapter VI

Differences in systemic and mucosal SARS-CoV-2 antibody prevalence in a prospective cohort of Dutch children

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ABSTRACT

Background

As SARS-CoV-2 will likely continue to circulate, low-impact methods become more relevant to monitor antibody-mediated immunity. Saliva sampling could provide a non-invasive method with reduced impact on children. Studies reporting on the differences between systemic and mucosal humoral immunity to SARS-CoV-2 are inconsistent in adults and scarce in children. These differences may be further unraveled by exploring associations to demographic and clinical variables.

Methods

To evaluate the use of saliva antibody assays, we performed a cross-sectional cohort study by collecting serum and saliva of 223 children attending medical services in the Netherlands (irrespective of SARS-CoV-2 exposure, symptoms or vaccination) from May to October 2021. With a Luminex and a Wantai assay, we measured prevalence of SARS-CoV-2 spike (S), receptor binding domain (RBD) and nucleocapsid-specific IgG and IgA in serum and saliva and explored associations with demographic variables.

Findings

The S-specific IgG prevalence was higher in serum 39% (95% CI 32 – 45%) than in saliva 30% (95% CI 24 – 36%) ($P \leq 0.003$). Twenty-seven percent (55/205) of children were S-specific IgG positive in serum and saliva, 12% (25/205) were only positive in serum and 3% (6/205) only in saliva. Vaccinated children showed a higher concordance between serum and saliva than infected children. Odds for saliva S-specific IgG positivity were higher in girls compared to boys (aOR 2.63, $P = 0.012$). Moreover, immunocompromised children showed lower odds for S- and RBD-specific IgG in both serum and saliva compared to healthy children (aOR 0.23 – 0.25, $P \leq 0.050$).

Conclusions

We showed that saliva-based antibody assays can be useful for identifying SARS-CoV-2 humoral immunity in a non-invasive manner, and that IgG prevalence may be affected by sex and immunocompromisation. Differences between infection and vaccination, between sexes and between immunocompromised and healthy children should be further investigated and considered when choosing systemic or mucosal antibody measurement.

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) will likely continue to circulate in the coming years. In the context of ongoing public health measures and vaccination programs, it is crucial to keep monitoring humoral immunity. Children have not been equally represented in immunosurveillance, while they do play a role in the transmission of SARS-CoV-2.[1] Surveillance of immunity in children is important to establish effective public health measures for this group. However, as the urgency of the pandemic decreases, it becomes more relevant to develop non-invasive methods to monitor antibody-mediated immunity to reduce impact on children and improve the willingness to participate. Coronavirus disease 2019 (COVID-19) convalescent patients and vaccinated individuals produce both serum and mucosal antibodies.[2, 3] Although serum antibodies are traditionally measured, saliva sampling for mucosal antibody measurements has shown promising first results leading to the first FDA approved saliva based antibody test in June 2021.[4, 5] As saliva sampling is quick and painless this could be a convenient alternative to serum sampling, in particular for children.

SARS-CoV-2 infection in the upper airway induces local innate and adaptive immune responses in the mucosa.[6] Evidence is growing that mucosal immunity, particularly through neutralizing antibodies, is important to control SARS-CoV-2 infection.[7-9] Mucosal and systemic immunity can function as separate compartments, but each can influence the other as well.[10, 11] As a result, both locally produced and systemically derived antibodies can be detected in the mucosa.[12] Although IgA is the most abundant isotype in mucosal surfaces, previous studies have supported the assumption that salivary IgG, which is mostly derived from the systemic compartment, is better suitable to detect previous SARS-CoV-2 exposure than salivary IgA.[13-15] The growing interest in mucosal immunity also stimulated the development of mucosal vaccines or therapeutic interventions, for which measuring the locally induced mucosal immune response will become even more important.[6, 16]

The development of mucosal assays or interventions is hampered by the lack of comprehensive understanding of mucosal immunity and its relation to systemic immunity.[17] Moreover, current literature comparing mucosal and systemic humoral immunity is inconsistent. While some studies have shown high prevalence of mucosal antibodies with similar durability to serum in convalescent patients,[15, 18] others reported lower proportions of patients with detectable mucosal antibodies as compared to serum.[19] In adult patients, quantity and durability of serum antibodies have been associated with sex or comorbidity.[20, 21] Evidence on associations of mucosal antibodies to sex, age or comorbidity is lacking or contradictory.[7, 14, 22] In addition, associations of humoral responses and demographic variables in children are rarely described. Differences in the induction and durability of systemic and mucosal humoral immunity may be unraveled by exploring these associations.

Prevalence studies among populations with a combination of natural and vaccine-induced immunity against SARS-CoV-2 are of value in evaluating the performance of mucosal antibody assays in the whole population. In our previous cohort during the first wave of the COVID-19 pandemic, we detected a low prevalence of SARS-CoV-2 specific IgG in children with differences in the presence of mucosal and systemic antibodies.[23] In the current prospective cross-sectional study, we describe higher prevalence for serum and saliva SARS-CoV-2 antibodies than in our previous cohort, and we further explore heterogeneity between serum and saliva by evaluating associations with demographic and clinical variables. We show that tracking humoral immunity through saliva-based assays could be useful for identifying SARS-CoV-2 naïve populations and vaccine responses.

MATERIALS AND METHODS

Study design

For this cross-sectional study we simultaneously sampled blood and saliva of children attending medical care at six secondary and tertiary care hospitals in the North-West region of the Netherlands during May 10th to October 15th 2021. All children aged 0 to 18 years requiring blood testing or intravenous cannulation for any reason were eligible. Eligibility was irrespective of a (suspected) acute or prior SARS-CoV-2 infection.

Study definitions

History of previous positive PCR- or rapid antigen test for SARS-CoV-2 infection and COVID-19 vaccination

status was collected to distinguish population subgroups. During the inclusion period of this study, the Health Council of the Netherlands announced their recommendation for COVID-19 vaccination in children ≥ 12 years of age with comorbidity. For analysis between population subgroups, inclusion 14 days after infection or vaccination was considered sufficient for a detectable antibody response.[9, 24] Children infected or vaccinated within 14 days prior to inclusion or with both a previous infection and vaccination were excluded from analysis within the population subgroups. Severity of SARS-CoV-2 infection was classified into five categories (asymptomatic, mild, moderate, severe and critically severe) of COVID-19 as published by Dong et al. or Multi-Inflammatory Syndrome in Children (MIS-C) based on patient-reported clinical features.[25] Children with immunodeficiency, autoimmune disease, hematological malignancy and/or use of immunomodulating drugs were defined as having an 'immunocompromised state'. Immunomodulating drugs included: azathioprine, methotrexate, monoclonal antibodies, immunoglobulins and corticosteroids. We defined children with an 'underlying illness' as children with obesity, respiratory, cardiovascular, endocrine, metabolic, hematologic, or kidney diseases, solid malignancies, or psychomotor retardation. Previously healthy children were categorized as having 'no relevant medical history'. Obesity was defined for children aged 2 to 5 years as weight-for-length z-score + 3 standard deviations (SD) and for children aged 5 to 18 years as BMI-for-age z-score + 2 SD.[26]

Sampling collection

Methods of serum and saliva sampling and analyses were as previously described.[23] In short, during venipuncture a blood sample of 1 to 5 ml was collected, centrifuged and serum was stored at -20° C. Saliva was obtained by passive drooling directly into a sterile container or via a buccal swab (ORACOL Saliva Collection Device, Product Code S10, Malvern Medical Developments Ltd) from which the saliva was extracted into a sterile tube by centrifugation. The resulting samples were centrifuged at 1000 rpm for 10 minutes and stored at -80° C.

Luminex assays

A Luminex assay was developed to determine SARS-CoV-2 specific antibodies in serum and saliva as described previously.[23] SARS-CoV-2 spike (S), receptor binding domain of the spike (RBD) and nucleocapsid (N) antigens were covalently coupled to Luminex MagPlex beads. Fifteen of each SARS-CoV-2 antigen coupled bead per µl was incubated with 1:10,000 diluted serum or 1:10 diluted saliva at a 1:1 ratio and incubated overnight at 4°C. The next day, washing was followed by a two hour incubation with goat anti-human IgG-PE or goat anti-human IgA-PE (Southern Biotech). After washing, detection was performed on a MAGPIX instrument (Luminex). Read-out was expressed as the median fluorescence intensity (MFI) of at least 50 beads per antigen. Positive and negative control beads were included in every well. To control for variation between plates, positive and negative control sera or saliva samples were included on every plate as well as a titration of serum or saliva of a known SARS-CoV-2 infected patient. The cut-offs for IgG antibody prevalence in each assay were established previously 23 and were further supported by testing serum of pre-pandemic (n = 113) or PCR-confirmed SARS-CoV-2 infected adults (n = 282) and testing pre-pandemic saliva samples of children (n = 50) or SARS-CoV-2 infected adults (n = 70) resulting in the sensitivity and specificity values presented in supplementary table 1. For IgA antibodies in saliva, the previously determined cut-offs were unsuitable due to low sensitivity. Instead, cut-offs were selected after ROC analysis, using pre-pandemic saliva samples of children (n = 50) and SARS-CoV-2 infected adults (n = 70), as the highest sensitivity achievable with a specificity of at least 80% (supplementary figure 1).

Additionally, we measured serum prevalence with the FDA approved Wantai SARS-CoV-2 RBD total antibody enzyme-linked immunosorbent assay to assess comparability between assays and between the prevalence in this study and other studies. Assays were performed following the manufacturer's instructions, providing a sensitivity of 97% (95% confidence interval [CI], 83 - 99%) and specificity of 98% (95% CI, 91 - 99%).[27]

Statistics

At the start of inclusion of this cohort, an S-specific total Ig seroprevalence of 32% was reported among Dutch adult blood donors during national surveys,[28] while seroprevalence among children was unknown. A minimum sample size of 214 participants was calculated to measure an expected S-specific IgG seroprevalence of 15% in our

cohort with a 95% CI between 10% and 20%.

All statistical analyses were performed in IBM SPSS Statistics version 26 predictive analytics software. Prevalence estimates were calculated as the proportion of participants of the total cohort with SARS-CoV-2 specific IgG above the cut-off for positivity. 95% CI was calculated with the Clopper-Pearson method.[29] T-tests and Mann-Whitney U tests were used to compare means and mean ranks across subgroups, and paired t-tests for comparisons of paired groups. Differences in proportion were tested with the Chi-square or Fisher's exact test and with McNemar test for paired proportions. Pearson correlation coefficients were determined for time since infection and antibody levels in serum and saliva and Spearman's rank-order correlations for correlations between serum and saliva antibodies. To study the associations between demographic and clinical variables and log transformed serum and saliva SARS-CoV-2 specific IgG , linear regression was performed only for children with antibody levels above the detection limit. Uni- and multivariable logistic regression were performed with serum and saliva SARS-CoV-2 specific IgG antibody prevalence and demographic or clinical variables. Cases with missing data for variables in the regression analysis were excluded. We identified several pre-defined demographic (age, sex) and clinical (comorbidity, COVID-19 vaccination and history of PCR or rapid antigen test positive infection) variables with clinical importance and/or a $P < 0.250$ in univariable regression analysis. These were included in the models after checking for multicollinearity using Variance Inflation Factors. Data are described as unadjusted and adjusted odds ratios (OR) with 95% CI. In antibody analyses of COVID-19 vaccinated children, only S- and RBD-specific IgG are reported with exclusion of N-specific IgG since N-specific IgG is not induced after vaccination.

Study approval

The study protocol was approved by the ethics committee of the Amsterdam University Medical Centers (NL73556.018.20). We obtained written informed consent from parents/guardians and/or from children above the legal age of consent.

Role of the funding source

The funding source stated in the acknowledgement section have financially supported the use of the assays in this study. The funding source did not have a role in the analysis or interpretation of the data, nor in developing and submitting the manuscript.

RESULTS

Study participants

Characteristics of the 223 included children are shown in table 1. Paired serum and saliva samples were available for 205 participants. Median age was 13 years with a range of 0 to 18 years and 50% of all children were female. Most children had an immunocompromised state (58%) while 27% reported another underlying illness and 15% reported no relevant medical history.

	Total cohort	Unknown exposure group	Infected group	Vaccinated group
Total N	**223**	**155**	**27**	**26**
Serum samples	212	147	26	24
Saliva samples	216	149	26	26
Sex				
Female	112 (50%)	69 (45%)	17 (63%)	17 (65%)
Male	111 (50%)	86 (55%)	10 (37%)	9 (35%)

	Total cohort	Unknown exposure group	Infected group	Vaccinated group
Age (years)				
< 1	9 (4%)	8 (5%)	0	0
1 - 4	13 (6%)	11 (7%)	2 (7%)	0
5 - 9	39 (18%)	34 (22%)	5 (19%)	0
10 - 14	77 (35%)	62 (40%)	5 (19%)	8 (31%)
15 - 17	85 (38%)	40 (26%)	15 (56%)	18 (69%)
Inclusion month				
May	34 (15%)	28 (18%)	5 (19%)	0
June	86 (39%)	65 (42%)	15 (56%)	2 (8%)
July	17 (8%)	12 (8%)	1 (4%)	1 (4%)
August	34 (15%)	19 (13%)	2 (8%)	11 (42%)
September	40 (18%)	25 (16%)	3 (11%)	7 (27%)
October	12 (5%)	6 (4%)	1 (4%)	5 (19%)
Immunocompromised state	**130 (58%)**	**84 (54%)**	**16 (59%)**	**18 (70%)**
Immunodeficiency	4 (2%)	4 (3%)	0	0
Autoimmune disease	125 (56%)	79 (51%)	16 (59%)	18 (69%)
Hematological malignancies	2 (1%)	2 (1%)	0	0
Use of immunomodulating drugs	131 (59%)	84 (54%)	16 (59%)	19 (73%)
Underlying illness	**59 (27%)**	**42 (27%)**	**8 (30%)**	**7 (27%)**
Obesity	21 (9%)	12 (9%)	2 (7%)	5 (19%)
Respiratory	5 (2%)	3 (2%)	2 (7%)	0
Cardiovascular	6 (3%)	2 (1%)	1 (4%)	2 (8%)
Neurological	5 (2%)	5 (3%)	0	0
Hematologic	10 (5%)	10 (7%)	0	0
Kidney disease	11 (5%)	6 (4%)	2 (7%)	1 (4%)
Endocrine/metabolic	11 (5%)	8 (5%)	1 (4%)	2 (8%)
Other disease	6 (3%)	4 (3%)	1 (4%)	1 (4%)
No relevant medical history	**34 (15%)**	**29 (19%)**	**3 (11%)**	**1 (4%)**
COVID-19 vaccination				
Not vaccinated	182 (82%)	154 (100%)	26 (100%)	0
Received 1 dose	19 (9%)	0	0	8 (31%)
Received 2 doses	19 (9%)	0	0	18 (69%)
Data missing	3 (1%)	N/A	N/A	N/A
Type of hospital visit				
Day-care	183 (82%)	122 (79%)	23 (85%)	24 (92%)
Outpatient	22 (10%)	18 (11%)	2 (7%)	2 (8%)
ER visit	4 (2%)	3 (2%)	1 (4%)	0
Inpatient	14 (6%)	12 (8%)	1 (4%)	0

Type of hospital				
University hospital	182 (82%)	126 (81%)	22 (81%)	23 (88%)
Non-university hospital	41 (18%)	29 (19%)	5 (19%)	3 (12%)
History of PCR proven SARS-CoV-2 infection				
Yes	34 (15%)	0	27 (100%)	0
No	163 (73%)	132 (85%)	0	26 (100%)
Clinical symptoms but tested negative or not tested	11 (5%)	9 (6%)	0	0
Data missing	15 (7%)	0	0	0
Hospital admission				
Yes	1 (0.4%)	N/A	1 (4%)	N/A
No	216 (97%)		20 (74%)	
ICU admission				
Yes	1 (0.4%)	N/A	1 (4%)	N/A
No	217 (97%)		26 (96%)	
SARS-CoV-2 infection severity	**N= 34**	**N/A**	**N= 27**	**N/A**
Asymptomatic	6		5	
Mild	19		14	
Moderate	1		1	
Severe	0		0	
Critically severe	1		1	
Data missing	7		5	
COVID-19 in household				
Yes	40 (18%)	17 (12%)	16 (59%)	3 (12%)
No	181 (81%)	137 (88%)	11 (41%)	23 (85%)

Table 1 Study population characteristics
Characteristics are described for the total cohort (n = 223) and for each study population subgroup. Abbreviations: COVID-19 = coronavirus disease 2019, ER = emergency room, ICU = intensive care unit, N/A = not applicable, N. = number, PCR = polymerase chain reaction assay, SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2.

Data on history of SARS-CoV-2 infection was available for 93% (208/223) of the total cohort. A positive PCR- or rapid antigen test for SARS-CoV-2 at least 14 days prior to inclusion was reported in 16% (34/208) of children. Of the SARS-CoV-2 infected children, 6/34 reported an asymptomatic infection, 19/34 a mild infection, 1/34 a moderate infection and 1/34 was a MIS-C patient. SARS-CoV-2 related hospital admission was reported for the MIS-C patient only, who did not require respiratory support but did require ICU admission for circulatory support. Another 5% (11/208) reported a suspicion of COVID-19 but had not been tested or was tested negative. One child received one vaccination prior to infection. Vaccination status was available for 99% (221/223). Of these 221 patients, 18% was vaccinated (6% received one dose at least 14 days prior to inclusion and 9% had received two doses). The median time since previously reported SARS-CoV-2 infection was six months (176 days) and median time since last COVID-19 vaccination was one month (30 days).

Prevalence and levels of S, RBD and N-specific antibodies

With the FDA approved Wantai assay, seroprevalence was 36% (75/209, 95% CI 29 – 43%, figure 1) for all participants. The Wantai RBD total antibody assay and the Luminex assay for serum RBD-specific IgG were in high agreement (96%); for further comparison of antibody levels and prevalence only Luminex serum assay results are reported. Levels of IgG and IgA antibodies in the Luminex assay are reported in supplementary figure 2. We observed heterogeneity in the S- RBD- and N-specific IgG prevalence: seroprevalence was higher for S-specific IgG; 39% (82/212, 95% CI 32 – 45%) and RBD-specific IgG; 38% (80/212, 95% CI 31 – 44%) compared to N-specific IgG; 18% (38/212, 95% CI 13 – 24%) in the Luminex assay (figure 1) (P < 0.001). In saliva, we similarly observed heterogeneity, as the antibody prevalence was 30% (64/216, 95% CI 24 – 36%), 25% (53/216, 95% CI 19 – 31%) and 13% (29/216, 95% CI 9 – 19%) for S-, RBD- and N-specific IgG, respectively. This was also significantly lower for N-specific IgG compared to S- and RBD-specific IgG (P < 0.001, figure 1).

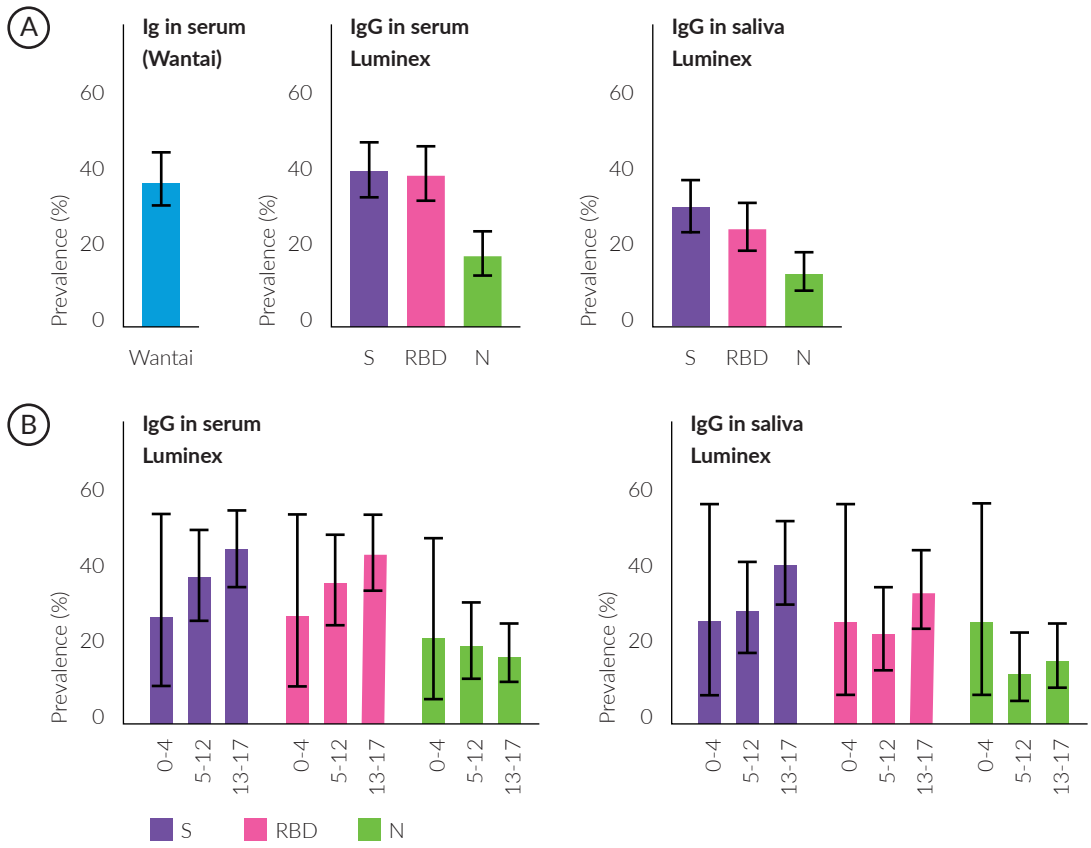

Figure 1 Prevalence of SARS-CoV-2 specific antibodies in serum and saliva

(A) Prevalence estimates of RBD-specific antibodies in serum using the Wantai assay (n = 209) and of S-, RBD- and N-specific antibodies using the Luminex assay in serum (n = 212) and saliva (n = 216) for all children (total cohort n = 223). (B) Prevalence of S-, RBD- and N-specific antibodies in all children, shown separately for pre-school children (0-4 years old, n = 19 for serum, n = 18 for saliva), primary school children (5-12 years old, n = 78 for serum, n = 82 for saliva) and secondary school children (13-17 years old, n = 115 for serum, n = 116 for saliva). Prevalence estimates are the calculated proportion with a value above the determined cut-off out. Estimates are shown with 95% confidence intervals. McNemar test was used for differences between paired proportions. Abbreviations: S = spike, RBD = receptor binding domain of the spike, N = nucleocapsid, *** = P ≤ 0.001.

Since N-specific IgG is only elicited by infection and not by vaccination, we additionally evaluated this heterogeneity in only unvaccinated children. The difference between N-specific IgG compared to S- and RBD-specific IgG was similarly significant in serum ($P < 0.008$), but not in saliva ($P > 0.050$, supplementary figure 3). In line with our previous study findings, sensitivity and specificity of saliva IgA was lower than saliva IgG to detect positive and negative control samples (supplementary figure 1). Since the highest sensitivity achievable with a specificity of at least 80% was 45 – 75% for SARS-CoV-2 specific IgA in saliva, we did not calculate prevalence for IgA antibodies. Antibody prevalence increased only for S- and RBD-specific IgG with age in the total cohort, although differences were not significant and absolute numbers for pre-school children were low (figure 1B). To further investigate the observed difference between S-, RBD- and N-specific antibody prevalence, we evaluated antibody levels over time since infection for children with a SARS-CoV-2 infection at least 14 days prior to inclusion (n = 27, figure 2). There was a decreasing trend for N-specific antibodies up to 432 days after infection, although there were no significant correlations between time and any antigen-specific antibody. Antibody levels were not assessed over time for the vaccinated subgroup due to the recent timing of vaccinations.

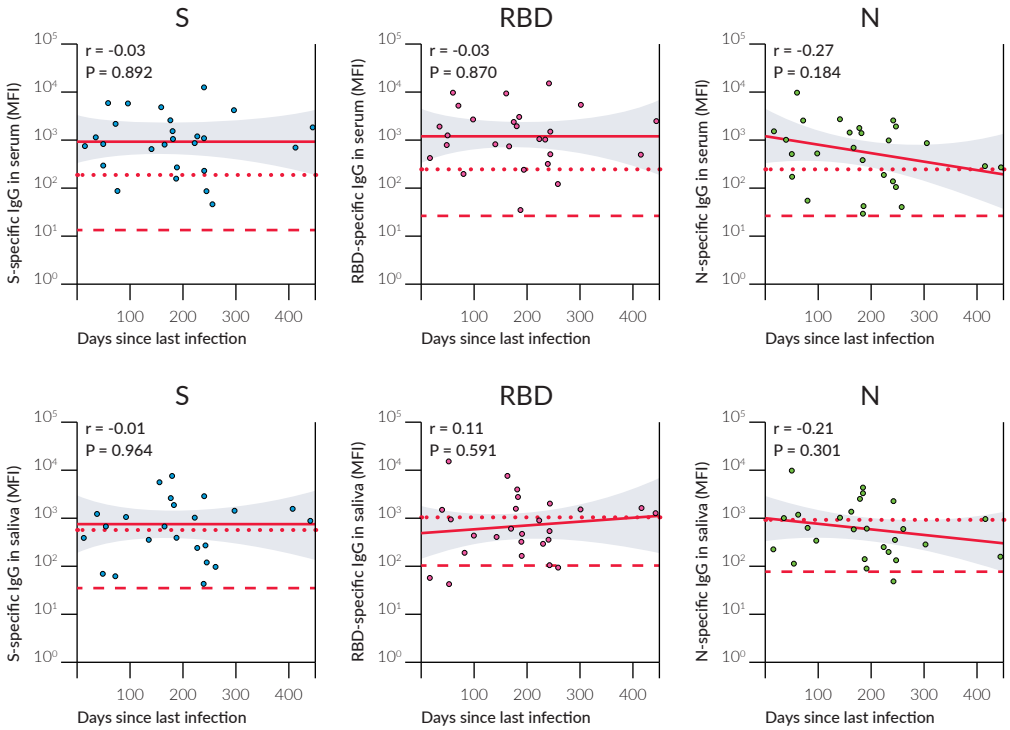

Figure 2 Antibody levels over time for different antigens in serum and saliva after infection
Levels of S-, RBD- and N-specific antibodies in serum (n = 26) and saliva (n = 26) of unvaccinated children with confirmed SARS-CoV-2 infection and known time of infection (total n = 27). Within each graph, each data point is a different individual. The solid red line represents a linear regression and the grey area the 95% confidence intervals. Pearson correlations were performed and the coefficient (r) and the p-value are shown for each graph. The dotted line indicates the assay cut-off and the dashed line represents the median MFI of all children in the unknown exposure group as a reference. Abbreviations: S = spike, RBD = receptor binding domain of the spike, N = nucleocapsid, MFI = median fluorescence intensity.

Comparison of serum and saliva IgG antibody prevalence and levels

We compared the IgG prevalence in all paired serum and saliva samples and detected a significantly lower prevalence in saliva for S- and RBD-specific IgG (P ≤ 0.003), while this was not significantly different for N-specific IgG (P = 0.082, figure 3). When evaluating the concordance between serum and saliva for all three antigens, 20-27% (42-55/205) of children was positive for both serum and saliva SARS-CoV-2 specific IgG, while 12-18% of children was only positive in serum.

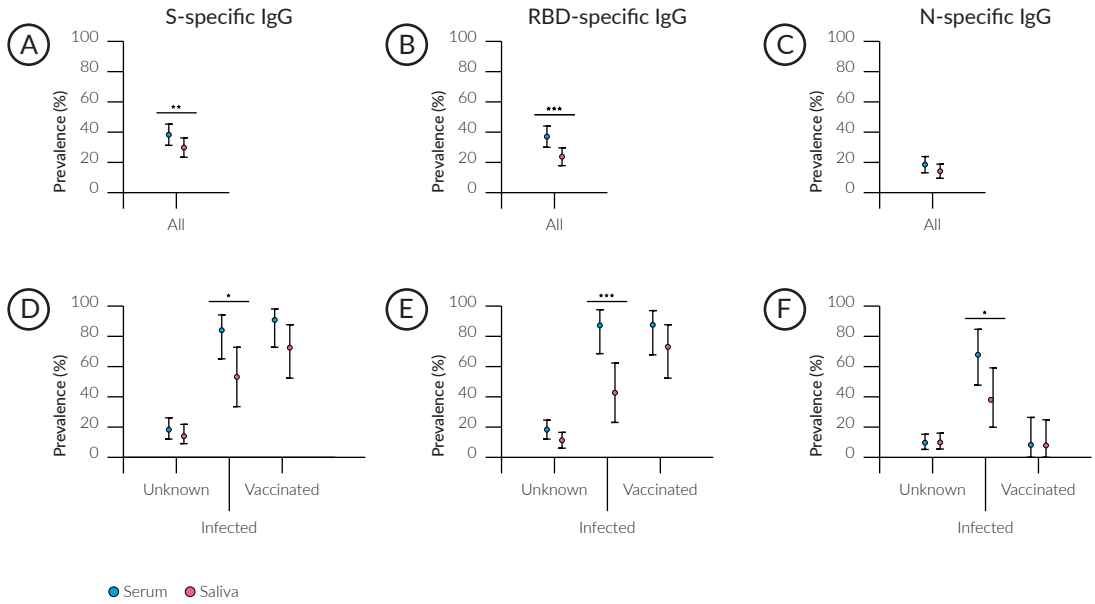

Figure 3 Prevalence of SARS-CoV-2 specific antibodies in serum and saliva compared for population subgroups
Prevalence estimates of antibodies in serum and saliva compared in the total cohort (serum n = 212 saliva n = 216 specific for (A) Spike, (B) RBD and (C) Nucleocapsid, and prevalence shown separately for the unknown group (serum n = 147, saliva n = 149), the infected group (serum n = 24, saliva n = 26) and the vaccinated group (serum n = 26, saliva n = 26) specific for (D) Spike, (E) RBD and (F) Nucleocapsid. Prevalence estimates are the calculated proportion with a value above the determined cut-off. Estimates are shown with 95% confidence intervals. McNemar test was used for differences between paired proportions. Abbreviations: S = spike, RBD = receptor binding domain of the spike, N = nucleocapsid, * = p < 0.050, ** = p ≤ 0.010, *** = p ≤0.001.

SARS-CoV-2-specific IgG in saliva could be detected in 54-69% of seropositive children (figure 4). Only 3% (6-6/223) of the cohort was positive in saliva while negative in serum. Further describing this group, saliva SARS-CoV-2 specific IgG was detected in 11% (15/134) of Wantai serum assay negative children (7% of total cohort) and 7% (9/131) of Luminex serum assay negative children (4% of the total cohort). Most of these children reported clinical clues for exposure to SARS-CoV-2 in the form of multiple positive saliva assays, history of vaccination or PCR positive infection, PCR positive infected household members or a combination (5%, 12/223 of the total cohort).

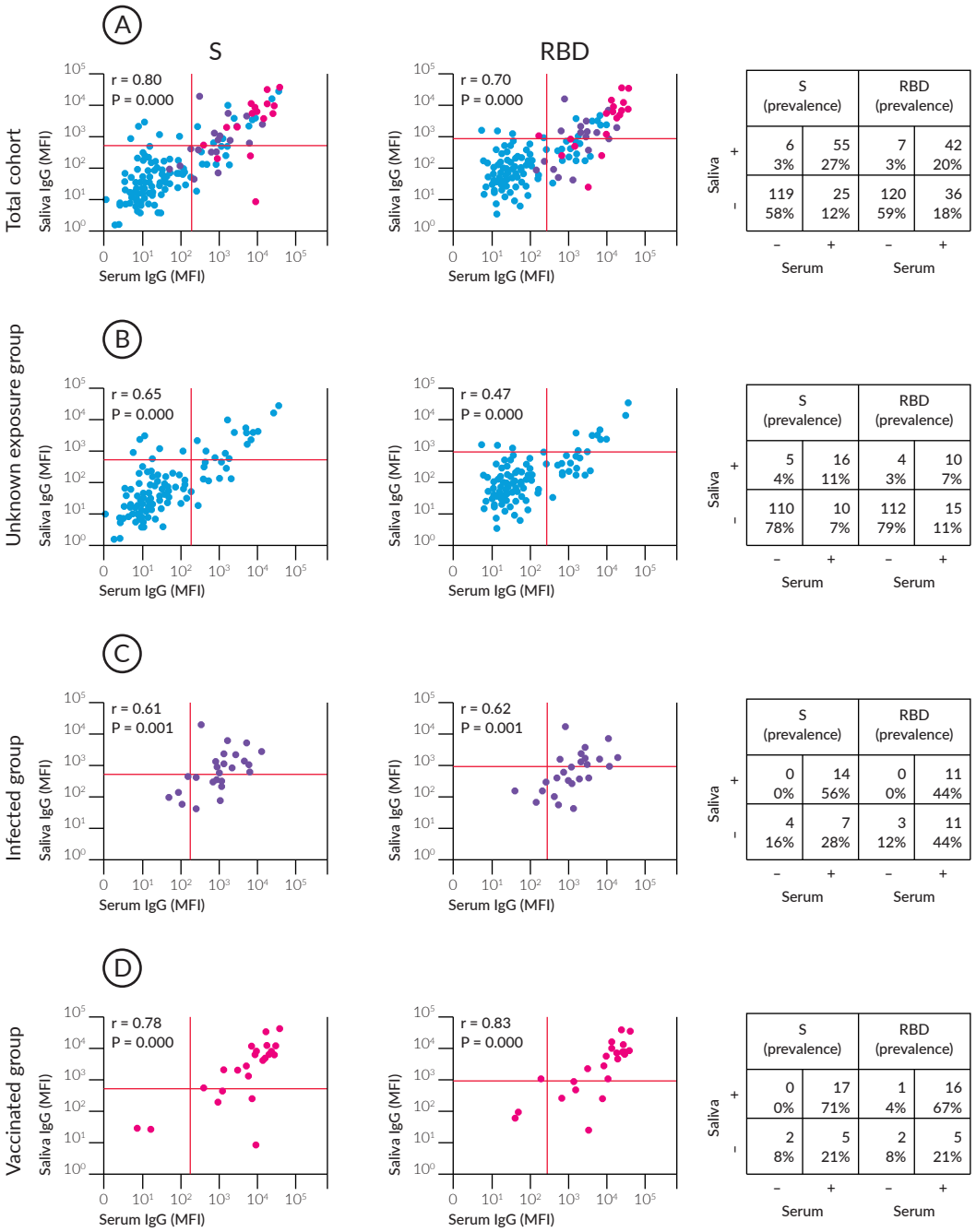

Figure 4 Comparison of serum and saliva antibody levels and prevalence

Levels and prevalence of S- and RBD-specific antibodies of children with paired samples of (A) the total cohort (n = 194) (B) the unknown exposed group (n = 141), (C) the infected group (n = 25) and (D) the vaccinated group (n = 24) in serum and saliva (shown on the x and y axis, prevalence is indicated by the percentages). The red line represents the cut-off for each assay. Spearman's rank correlations were performed and the coefficient (r) and the p-value are shown for each graph. Abbreviations: S = Spike, RBD = receptor binding domain of the spike, MFI = median fluorescence intensity.

To evaluate the correlation between serum and saliva antibodies, we calculated Spearman's rank-order correlations. We observed a strong positive correlation between serum and saliva for S- and RBD- specific IgG (r 0.80, P < 0.001 and r 0.70, P < 0.001 respectively, figure 4). For N-specific IgG, which is only elicited by infection and not by vaccination, we found a moderate positive correlation between serum and saliva (r 0.49, P < 0.001, supplementary figure 4). Saliva IgA only correlated weakly with serum IgG for S-specific antibodies (r 0.3, P < 0.001, supplementary figure 5).

Comparison of serum and saliva IgG antibodies in population subgroups

For subgroup analyses, the study cohort was divided into three groups: the infected group (n= 27), only consisting of children with a history of PCR- or rapid antigen test confirmed SARS-CoV-2 infection at least 14 days prior to study inclusion; the vaccinated group (n= 26), only consisting of all children that received at least one vaccination dose at least 14 days prior to study inclusion; and the unknown exposure group (n= 156), consisting of unvaccinated children with no known history of SARS-CoV-2 infection. Six children were both vaccinated and reported a history of SARS-CoV-2 infection and were excluded from subgroup analyses.

We investigated the serum and saliva antibody prevalence separately in the subgroups (figure 3). In the unknown exposure group there was only a significant difference between serum and saliva RBD-specific IgG prevalence (18% and 11% respectively, P = 0.019). In the infected group, there was a significant difference between serum and saliva IgG for all three antigens (P < 0.040). In the vaccinated group there was no significant difference between serum and saliva for all three antigens. The vaccinated group showed the highest concordance between serum and saliva with 67-71% (16-17/24) positive in both compartments (figure 4). In the infected group, 44 – 56% (11-14/25) of children was positive in both serum and saliva. We observed more children with S and RBD-specific serum IgG but no S and RBD-specific saliva IgG in the infected group compared to the vaccinated group (28 – 44% versus 21%, respectively). In all subgroups, only a small proportion (0-4%) was only positive in saliva while negative in serum. Spearman's rank-order correlations between serum and saliva S-specific IgG were stronger in the vaccinated group compared to the infected group (r = 0.78 versus r = 0.61, respectively, figure 4).

Associations with demographic and clinical variables

Since prevalence of saliva SARS-CoV-2 IgG was lower compared to serum, we investigated if prevalence was associated with sex, age or comorbidity in the total cohort using a multivariable logistic regression model adjusting also for vaccination and history of infection confirmed by PCR or rapid antigen test. Prevalence of saliva S-specific IgG was higher in girls (40%) compared to boys (19%, P < 0.02). In the multivariable analysis correcting for age, comorbidity, vaccination and infection, sex was a significant predictor for S-specific IgG prevalence in saliva (aOR 2.63, 95% CI 1.24 – 5.58) but not for RBD-specific IgG in saliva nor for S- and RBD-specific IgG in serum (table 2). There was an age-related association with saliva and serum SARS-CoV-2 specific IgG which disappeared after correcting for sex, comorbidity, vaccination and infection. Regarding comorbidity, lower odds for RBD-specific IgG positivity in saliva and S- and RBD-specific IgG in serum were seen for immunocompromised compared to healthy children (aOR 0.23 – 0.25, P < 0.050, table 2). When evaluating associations of variables with SARS-CoV-2 specific IgG in linear regression, sex, age and comorbidity were not associated (data not shown).

Saliva S-specific IgG		Univariable			Multivariable	
	OR	95% CI	P	aOR	95% CI	P
Female sex	2.82	1.53 – 5.20	**< .001**	2.63	1.24 – 5.58	**0.012**
Age	1.10	1.02 – 1.18	**0.013**	1.05	0.95 – 1.16	0.350
No comorbidity	Ref			Ref		
Immunocompromised	1.06	0.44 – 2.61	0.891	0.27	0.08 – 1.01	0.051
Other illness	1.49	0.56 – 3.94	0.426	0.80	0.24 – 2.64	0.718
No COVID-19 vaccination	Ref			Ref		
One dose received	5.31	1.60 – 17.65	**0.007**	5.01	1.26 – 19.93	**0.022**
Two doses received	32.21	7.13 – 145.54	**< .001**	43.74	8.83– 216.76	**< .001**
Previous SARS-CoV-2 infection	4.48	2.05 – 9.79	**< .001**	5.87	2.40 – 14.39	**< .001**

Saliva RBD-specific IgG		Univariable			Multivariable	
	OR	95% CI	P	aOR	95% CI	P
Female sex	2.21	1.16 – 4.19	**0.015**	2.10	0.96 – 4.61	0.064
Age	1.07	0.99 – 1.15	0.086	1.03	0.92 – 1.14	0.630
No comorbidity	Ref			Ref		
Immunocompromised	0.87	0.34 – 2.24	0.773	0.25	0.06 – 0.99	**0.048**
Other illness	1.64	0.60 – 4.51	0.335	1.00	0.30 – 3.36	0.998
No COVID-19 vaccination	Ref			Ref		
One dose received	5.07	1.53 – 16.78	**0.008**	5.70	1.44 – 22.64	**0.013**
Two doses received	27.02	7.41 – 98.54	**< .001**	41.91	10.06 – 174.68	**< .001**
Previous SARS-CoV-2 infection	2.87	1.31 – 6.28	**0.008**	4.59	1.82 – 11.57	**0.001**

Serum S-specific IgG		Univariable			Multivariable	
	OR	95% CI	P	aOR	95% CI	P
Female sex	2.17	1.24 – 3.83	**0.007**	1.82	0.86 – 3.83	0.116
Age	1.10	1.03 – 1.17	**0.007**	1.08	0.98 – 1.19	0.133
No comorbidity	Ref			Ref		
Immunocompromised	1.02	0.45 – 2.32	0.962	0.24	0.07 – 0.80	**0.020**
Other illness	1.62	0.65 – 4.04	0.298	1.03	0.34 – 3.10	0.957
No COVID-19 vaccination	Ref			Ref		
One dose received	25.67	3.23 – 203.77	**0.002**	32.26	3.56 – 292.73	**0.002**
Two doses received	37.33	4.83 – 288.64	**< .001**	58.52	7.05 – 485.74	**< .001**
Previous SARS-CoV-2 infection	16.20	5.42 – 48.45	**< .001**	22.96	6.98 – 75.54	**< .001**

Serum RBD-specific IgG	Univariable			Multivariable		
	OR	95% CI	P	aOR	95% CI	P
Female sex	2.02	1.15 – 3.55	**0.015**	1.56	0.73 – 3.32	0.249
Age	1.09	1.02 – 1.17	**0.009**	1.07	0.97 – 1.19	0.169
No comorbidity	Ref			Ref		
Immunocompromised	0.99	0.43 – 2.24	0.973	0.23	0.07 – 0.79	**0.019**
Other illness	1.51	0.60 – 3.75	0.380	0.93	0.30 – 2.82	0.893
No COVID-19 vaccination	Ref	Ref		Ref		
One dose received	11.98	2.54 – 56.54	**0.002**	14.44	2.54 – 81.96	**0.003**
Two doses received	38.34	4.96 – 296.50	**< .001**	64.98	7.81 – 540.76	**< .001**
Previous SARS-CoV-2 infection	24.26	7.08 – 83.19	**< .001**	35.95	9.63 – 134.22	**< .001**

Table 2 Logistic regressions for serum and saliva IgG prevalence
Uni- and multivariable regression values with serum and saliva IgG levels above the cut-off for positivity as the dependent variable. Odds ratios (OR) and adjusted odds ratios (aOR) with 95% confidence intervals (CI) for variables included in the regression models are reported. Variables reaching statistical significance are presented in bold ($p < 0.050$). Study inclusion at least 14 days after PCR- or rapid antigen test confirmed SARS-CoV-2 infection or first dose of COVID-19 vaccination was considered sufficient for inclusion in the regression models. Abbreviations: COVID-19 = coronavirus disease 2019, PCR = polymerase chain reaction assay, S = Spike ,RBD = Receptor binding domain, SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2.

DISCUSSION

We detected SARS-CoV-2 specific IgG and IgA in both serum and saliva in a substantial group of children attending regular medical services. There was heterogeneity in the humoral response with an overall higher prevalence in serum compared to saliva. Vaccinated children showed higher correspondence between serum and saliva positivity than previously infected children. Moreover, girls had higher odds for saliva SARS-CoV-2 specific IgG compared to boys and immunocompromised children had lower odds for both serum and saliva IgG. Understanding the differences between systemic and mucosal humoral responses provides insight for the application of mucosal antibody assays.

Similar to our previous study, we observed that determining the prevalence for SARS-CoV-2 specific mucosal IgA was less accurate than for IgG, and that salivary IgA correlates poorly to serum IgA. This is consistent with other studies emphasizing the lower sensitivity for saliva IgA to detect PCR-positive patients, more nonspecific and cross-reactive binding of saliva IgA and a shorter durability as compared to saliva IgG.[13-15, 18, 30] The IgG antibody prevalence among Dutch children attending regular medical services has increased sharply from 3 – 4% in serum and saliva in our previous 2020 study to 30 – 38% in 2021.[23] In the corresponding period of 2021, seroprevalence among Dutch adult blood donors in a national survey increased from 31% to 95% due to vaccination programs and increasing infection rates. Compared to adults, children experience more frequent asymptomatic infections. With a large proportion of infections remaining asymptomatic, as was also seen in our cohort of children, antibody assays are an important addition to symptom driven PCR testing to assess infection rates and estimate immunity in a population.

We report heterogeneity between antibodies targeting different antigens, with a significantly lower prevalence for N-specific antibodies, also when analyzing only unvaccinated children. An important factor in explaining these

differences could be the variation in time kinetics. We potentially observed a more rapid decline over time for N-specific IgG compared to S and RBD-specific IgG, measured up to more than one year after infection. Several longitudinal studies corroborate a faster decline of N-specific IgG compared to S- and RBD-specific IgG, showing a significant drop in N-specific IgG several months after infection in serum and saliva.[31-33] Importantly, a study of antibody dynamics showed a several-fold variation between individuals in half-lives of SARS-CoV-2 specific IgG.[34] In addition, N-specific antibodies often seem to be absent in asymptomatic patients.[35, 36] Therefore, besides a more rapid decline of specific antibodies over time, there could be heterogeneity between individuals in the elicitation and preservation of specific humoral responses in the first place. In a broader perspective, an analysis of peripheral blood mononuclear cells showed substantial variability between healthy individuals in numbers of naïve B cells, plasmablasts, memory CD4+ T cells, effector CD8+ T cells and mucosa-associated innate T-cells, suggesting individual tendencies towards a more pronounced B-cell mediated or T-cell mediated response to pathogens.[37]

In line with our previous study, heterogeneity is also shown between the mucosal and systemic compartments.[23] Although serum and saliva IgG were both detectable in most SARS-CoV-2-specific IgG positive children, saliva IgG prevalence was lower than serum IgG prevalence. Longitudinal studies suggest a difference in time kinetics, with slightly lower percentages of saliva IgG positive individuals remaining positive after 9 to 15 months follow-up compared to serum IgG in mild adult COVID-19 patients (72 – 88% in saliva compared to 89 – 96% in serum).[15, 38] We also report a greater difference between serum and saliva IgG in the infected group as compared to the vaccinated group. Median time since last exposure or vaccination respectively was six-fold longer in the infected group as compared to the vaccinated group which is likely to contribute to this difference. Considering that some studies measure similar durability for serum and mucosal SARS-CoV-2 IgG,[39, 40] heterogeneity in the response itself could also explain the differences between compartments. If exposure to the virus does not elicit identical humoral immune responses in all individuals, this may explain the lower saliva prevalence reported in several COVID-19 cohorts.[19, 41]

To explore the value of mucosal samples, it is crucial to identify which factors can predict certain systemic or mucosal humoral responses. We showed an association between female sex and saliva SARS-CoV-2-specific antibody positivity in children. This association was not found for serum SARS-CoV-2-specific antibody positivity, suggesting that the mucosal compartment may be more prone to sex-related differences than the systemic compartment. Differences between male and female immunity – although predominantly after sexual maturation – have been described with stronger antibody responses, higher basal Ig titers and higher number of B cells in females.[42] Our lack of sexual development data, such as Tanner scores, therefore imposes an important limitation in evaluating associations with sex and this information should be collected in future pediatric antibody studies. In SARS-CoV-2 infection, adult males show a slower more gradual increase of RBD-specific IgG in the acute phase and a faster decline of S- and RBD-specific and neutralizing antibodies compared to females.[33, 43-45] Of note, in our study this association with female sex was only apparent in S-specific IgG but not in RBD- and N-specific IgG. This may be explained by slightly lower prevalence of RBD- and N- compared to S-specific IgG thus lacking sufficient numbers to reach statistical significance. Alternatively, associations of antibodies with sex and comorbidity could indeed be antigen-specific, and thus may only be present for S-specific IgG. Supporting this latter hypothesis, similar findings have been reported for convalescent patients showing significantly higher S1-specific antibody prevalence in females compared to males, whereas the difference between sexes in RBD- and N-specific antibody prevalence was not significant.[46] This possibility of antigen-specific associations should be taken into consideration in future studies. Moreover, we observed lower odds for immunocompromised compared to healthy children for saliva RBD-specific IgG and serum S- and RBD-specific IgG, indicating a possible higher risk for more frequent or severe reinfection. As immunocompromised patients can show adequate serum and saliva responses after two vaccinations, the clinical relevance of lower humoral responses is probably largely influenced by the type of disease or immunomodulating drugs, previous SARS-CoV-2 immunity and number and timing of vaccinations.[47, 48]
The relevance of non-invasive mucosal antibody assays is increasing in this phase of the pandemic because of the increasing vaccination rates and decreasing hospital admission rates. Described in this study and in several other

cohorts, the humoral response after vaccination shows a higher correlation between the mucosal and systemic humoral IgG responses when compared to natural infection.[3, 49, 50] This suggests a potential use for saliva antibodies to monitor vaccine response. Additionally, in line with our previous study we found Wantai seronegative children with mucosal antibodies. Considering the 96% specificity, this could be explained by possible false positivity. However, some COVID-19 cohorts have similarly shown patients with mucosal antibodies without seroconversion.[41, 51] Furthermore, most of our seronegative children with mucosal antibodies showed convincing clinical clues for SARS-CoV-2 exposure. Saliva assays are less sensitive than serum assays but they can potentially identify seronegative convalescent patients with saliva antibodies.

In children, this is the first study evaluating associations of mucosal antibodies and demographic variables. However, there are several limitations. An important limitation is the lack of complete data regarding exposure to SARS-CoV-2 as we did not perform structured PCR testing in the participants. By means of written and verbal questionnaires, we have questioned all participants whether they were aware of a previous SARS-CoV-2 infection. This study investigated associations of demographic variables with antibody positivity by including known previous PCR positivity and vaccination status in the models. In this way, we aimed to investigate differences in humoral responses instead of differences in exposure to SARS-CoV-2. The inclusion of previous infection and vaccination data in the regression models did indeed change the outcome. Unfortunately, data on COVID-19 severity was too scarce to include in the regression analyses. Finally, our study cohort included as large proportion of immunocompromised children and children using immunomodulating drugs, which allowed investigation of the effect of this variable, but is also a limitation for the general applicability of our results.

In conclusion, this cross-sectional study confirms that humoral immunity can be detected in saliva, preferably with S- and RBD-specific IgG. Sex and immunocompromisation may affect antigen-specific SARS-CoV-2 IgG antibody prevalence in children. Differences between infection and vaccination, between sexes and between immunocompromised and healthy children should be further investigated and considered when choosing systemic or mucosal antibody measurement. Future studies may also focus on longitudinal analysis of antibody levels in repeated saliva samples from children and the protective capacity of saliva antibodies. On a population level, saliva-based assays can be useful for identifying vulnerable SARS-CoV-2 naive populations and vaccine responses in a non-invasive manner.

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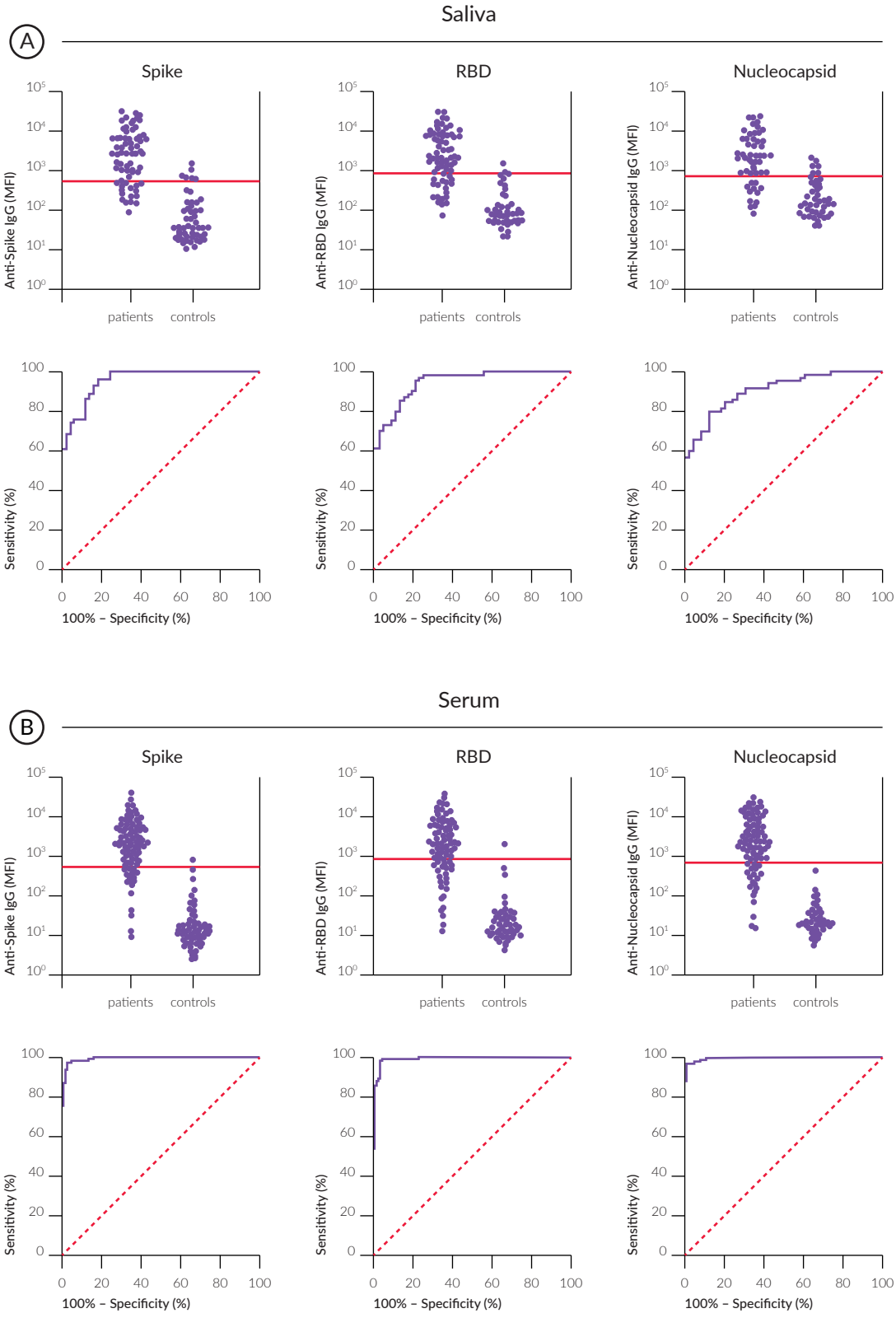
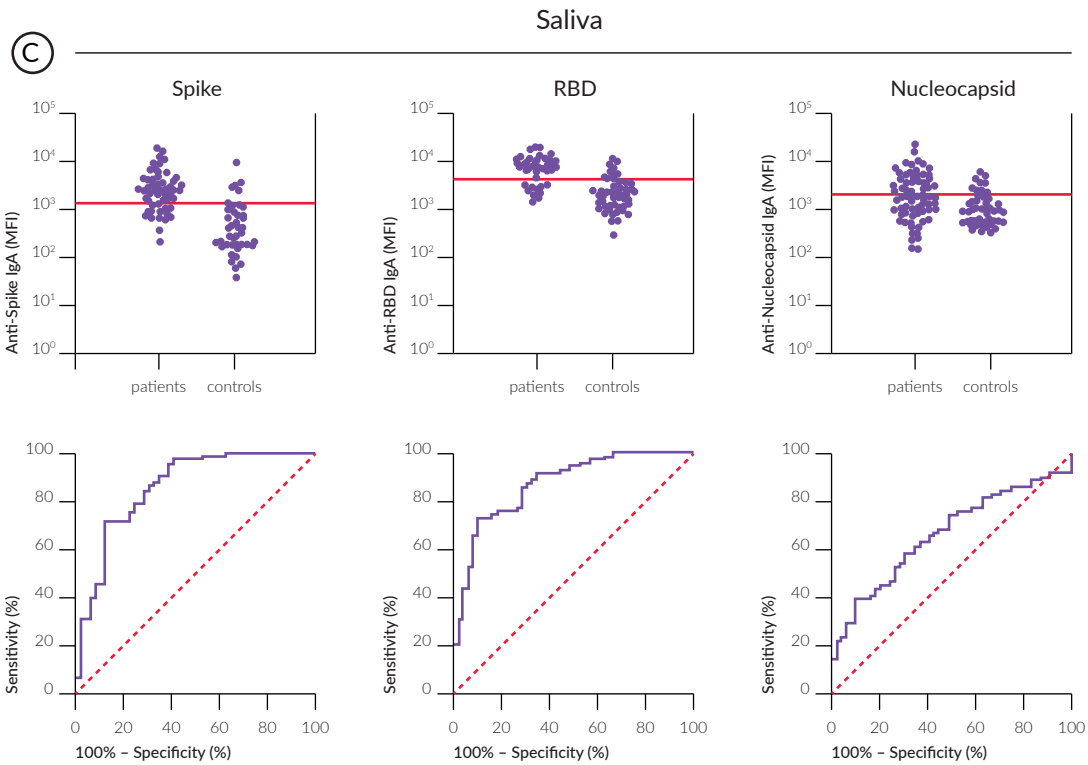
SUPPLEMENTARY MATERIALS

	Sensitivity	Sensitivity 95%CI	Specificity	Specificity 95%CI
Serum, S, IgG	97%	92.48% to 99.28%	96%	93.60% to 98.06%
Serum, RBD, IgG	96%	91.25% to 98.61%	91%	87.65% to 94.21%
Serum, N, IgG	99%	95.16% to 99.95%	94%	90.14% to 95.92%
Saliva, S, IgG	77%	66.05% to 85.41%	88%	76.20% to 94.38%
Saliva, RBD, IgG	73%	61.46% to 81.88%	96%	86.54% to 99.29%
Saliva, N, IgG	80%	69.18% to 87.70%	88%	76.20% to 94.38%
Saliva, S, IgA	71%	59,43% to 80,38%	88%	76,20% to 94,38%
Saliva, RBD, IgA	75%	64,04% to 84,01%	82%	69,20% to 90,23%
Saliva, N, IgA	45%	33,77% to 56,62%	80%	66,96% to 88,76%

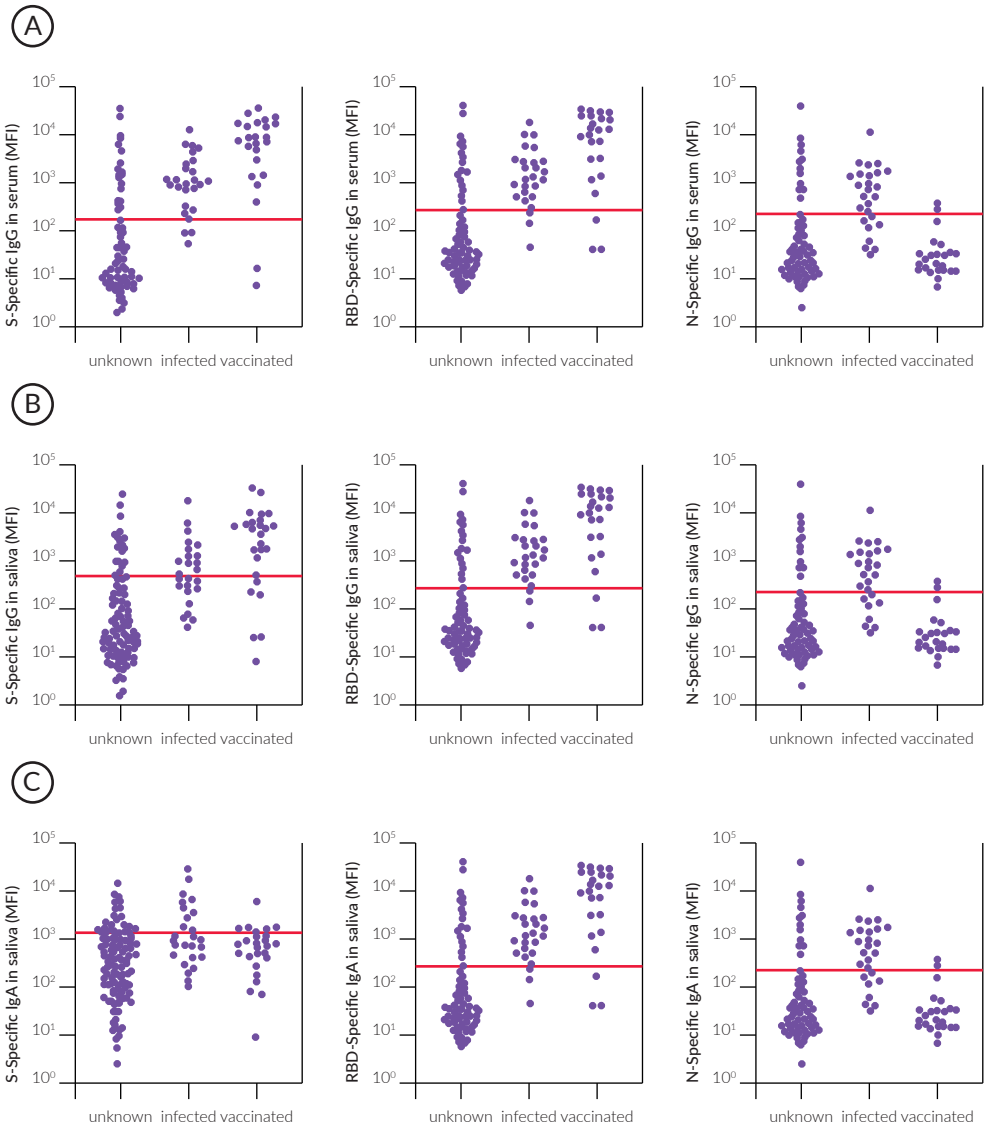
Supplementary table 1 Specificity and sensitivity of Luminex assays to detect previous PCR positive SARS-CoV-2 infection. Abbreviations: S = spike, RBD = receptor binding domain of the spike, N = nucleocapsid.


Saliva
A
Spike
RBD
Nucleocapsid
Anti-Spike IgG (MFI)
Anti-RBD IgG (MFI)
Anti-Nucleocapsid IgG (MFI)
patients
controls
Sensitivity (%)
100% – Specificity (%)
Serum
B
Spike
RBD
Nucleocapsid
Anti-Spike IgG (MFI)
Anti-RBD IgG (MFI)
Anti-Nucleocapsid IgG (MFI)
patients
controls
Sensitivity (%)
100% – Specificity (%)

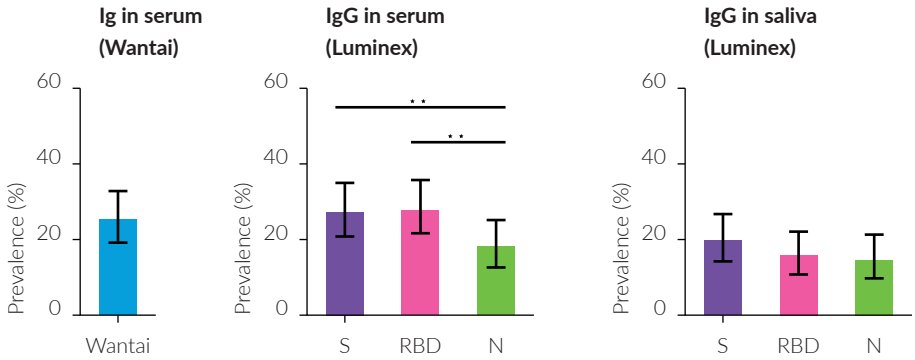


Supplementary Figure 1 ROC analysis for Luminex assays

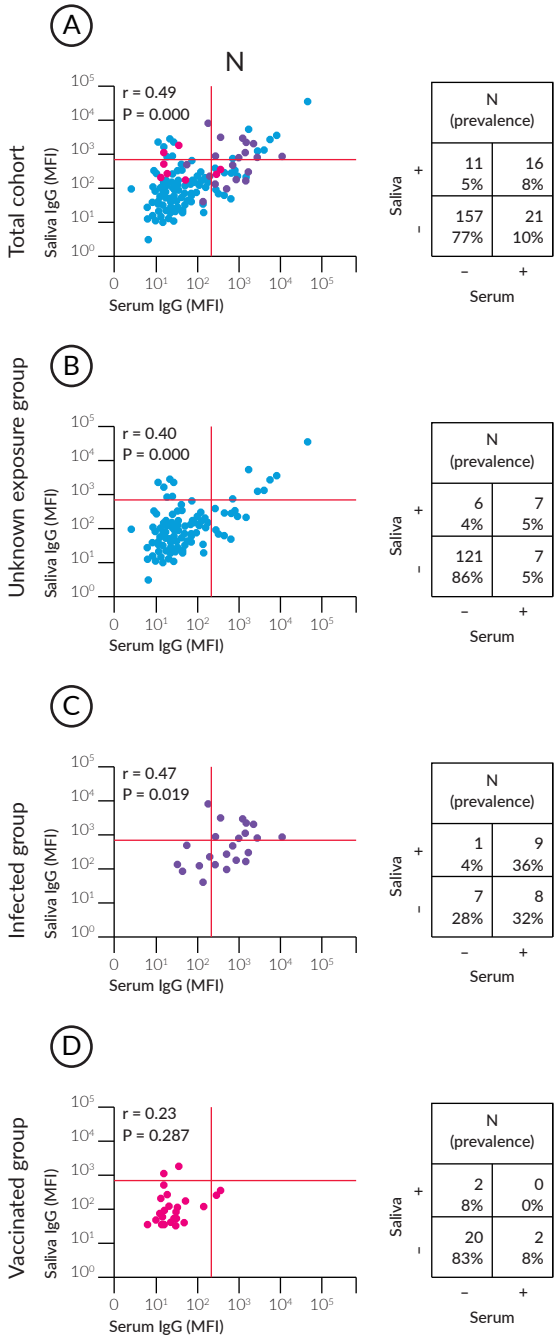
(A) ROC analysis corroborating our previously determined cut-off (ref KIDS 1) for the Luminex assay for IgG in saliva with pre-pandemic saliva of children (n = 50) and SARS-CoV-2 infected adults (n = 70). (B) ROC analysis corroborating our previously determined cut-off (ref KIDS 1) for the Luminex assay for IgG in serum with serum of pre-pandemic (n = 113) and PCR-confirmed SARS-CoV-2 infected (n = 282) adults. The red dotted lines are the cut-offs. (C) ROC analysis to determine a new cut-off for the Luminex assay for IgA in saliva using pre-pandemic saliva of children (n = 50) and SARS-CoV-2 infected adults (n = 70). Cut-offs were selected as the highest sensitivity achievable with a specificity of at least 80% and indicated as red dotted lines. Abbreviations: S = spike, RBD = receptor binding domain of the spike, N = nucleocapsid, MFI = median fluorescence intensity.



Supplementary Figure 2 Levels of SARS-CoV-2 specific IgG and IgA in serum and saliva
(A) Levels of S, RBD and N-specific IgG in serum, presented separately for the unknown exposure group (n = 147), the infected group (n = 26) and the vaccinated group (n = 24). (B) Levels of S-, RBD- and N-specific IgG in saliva, presented separately for the unknown exposure group (n = 149), the infected group (n = 26) and the vaccinated group (n = 26). (C) Levels of S-, RBD- and N-specific IgA in saliva, presented separately for the unknown exposure group (n = 149), the infected group (n = 26) and the vaccinated group (n = 26). The red lines are the cut-offs for positivity. Abbreviations: S = spike, RBD = receptor binding domain of the spike, N = nucleocapsid, MFI = median fluorescence intensity.

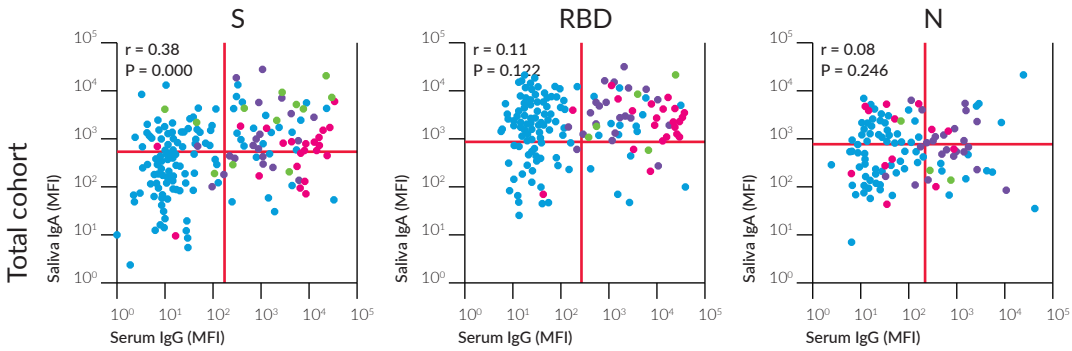


Supplementary Figure 3 Prevalence of SARS-CoV-2 specific antibodies in serum and saliva in unvaccinated children
Prevalence estimates of RBD-specific antibodies in serum using the Wantai assay (n = 171) and of S-, RBD- and N-specific antibodies using the Luminex assay in serum (n = 173) and saliva (n = 175) only for unvaccinated children (in total n = 182). Prevalence estimates are the calculated proportion with a value above the determined cut-off out. Estimates are shown with 95% confidence intervals. McNemar test was used for differences between paired proportions. Abbreviations: S = spike, RBD = receptor binding domain of the spike, N = nucleocapsid.



Supplementary Figure 4 Comparison of N-specific serum and saliva antibody levels and prevalence

Levels and prevalence of N-specific antibodies of children with paired samples of (A) the total cohort (n = 194) (B) the unknown exposed group (n = 141), (C) the infected group (n = 25) and (D) the vaccinated group (n = 24) in serum and saliva (shown on the x and y axis, prevalence is indicated by the percentages). The red line represents the cut-off for each assay. Spearman's rank correlations were performed and the coefficient (r) and the p-value are shown for each graph. Abbreviations: N = Nucleocapsid, MFI = median fluorescence intensity.



Supplementary Figure 5 Comparison of saliva IgA and serum IgG levels and prevalence

Levels and prevalence of S, RBD and N-specific antibodies of children with paired samples of the total cohort (n = 194) in serum and saliva (shown on the x and y axis, prevalence is indicated by the percentages). The red line represents the cut-off for each assay. Spearman's rank correlations were performed and the coefficient (r) and the p-value are shown for each graph. Abbreviations: S = spike, RBD = receptor binding domain of the spike, N = Nucleocapsid, MFI = median fluorescence intensity

2

Challenges and opportunities in fever without a source guidelines

Chapter VII

Fever without an apparent source in young infants: a multicenter retrospective evaluation of adherence to the Dutch guidelines

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ABSTRACT

Background

The Dutch fever without an apparent source (FWS) guidelines were published to timely recognize and treat serious infections. We determined the adherence to the Dutch FWS guidelines and the percentage of serious infections in infants younger than 3 months of age. Second, we identified which clinical criteria, diagnostic tests, and management were associated with nonadherence to the guidelines.

Methods

A retrospective cohort study was performed in 2 Dutch teaching hospitals. We assessed the charts of all infants with FWS who presented at the emergency departments from September 30, 2017, to October 1, 2019. Diagnostic and therapeutic decisions were compared with the recommendations, as published in the Dutch guidelines. Infants were categorized into the nonadherence group in case 1 or more recommendations were not adhered to.

Results

Data on 231 infants were studied; 51.5% of the cases adhered to the Dutch guidelines and 16.0% suffered from a serious infection. The percentage of infants with a serious infection was higher in the adherence compared with the nonadherence group. We observed no relevant differences in clinical outcomes. Univariate regression analysis showed that an abnormal white blood cell count was associated with nonadherence (OR 0.4, P = 0.049). Not obtaining a urine and blood culture and not starting intravenous antibiotic treatment were the most frequent reasons for nonadherence to the guidelines.

Conclusions

Our study indicates that there was nonadherence in a large proportion of FWS cases. The guidelines may need to be adjusted to increase adherence.

INTRODUCTION

Fever in infants younger than three months, is one of the most common reasons to visit the emergency department in high-income countries.[1,2] Most infants have a mild and clinically not harmful viral infection, whereas 6% to 15% suffer from a serious infection requiring immediate treatment. [2-7] However, the clinical presentation of these infections is often nonspecific. In addition, infants younger than three months are at greater risk to suffer from a more serious infection as compared to older infants. Due to that reason, the threshold to undergo invasive diagnostic testing and to receive intravenous antimicrobials is low in infants younger than three months suffering from fever without an apparent source (FWS).[8-10] Clinical decision rules have been developed to guide clinicians to recognize a serious infection in time and to treat it appropriately, aiming to reduce excessive diagnostic testing and treatment.[11-17] Studies on adherence and outcome of these existing decisions rules in clinical practice are scarce.[18-21] Evaluation of the use of clinical decision rules and the impact in clinical practice after implementation are essential for improving the quality of care.[22-24]

In the Netherlands, the national FWS guidelines adapted from the National Institute for Health and Clinical Excellence (NICE) were published in 2013.[10,25] The adherence to and appropriateness of these guidelines in clinical practice are unknown. Our primary aims were therefore to evaluate adherence to the Dutch guidelines and to compare the clinical outcomes in case of adherence compared to non-adherence to the guidelines. The secondary aims were to investigate which recommended diagnostics and management were not performed, and to determine which clinical criteria to classify for high risk of infection were predictive for adherence to the guidelines as well as to non-adherence.

METHODS

Study design

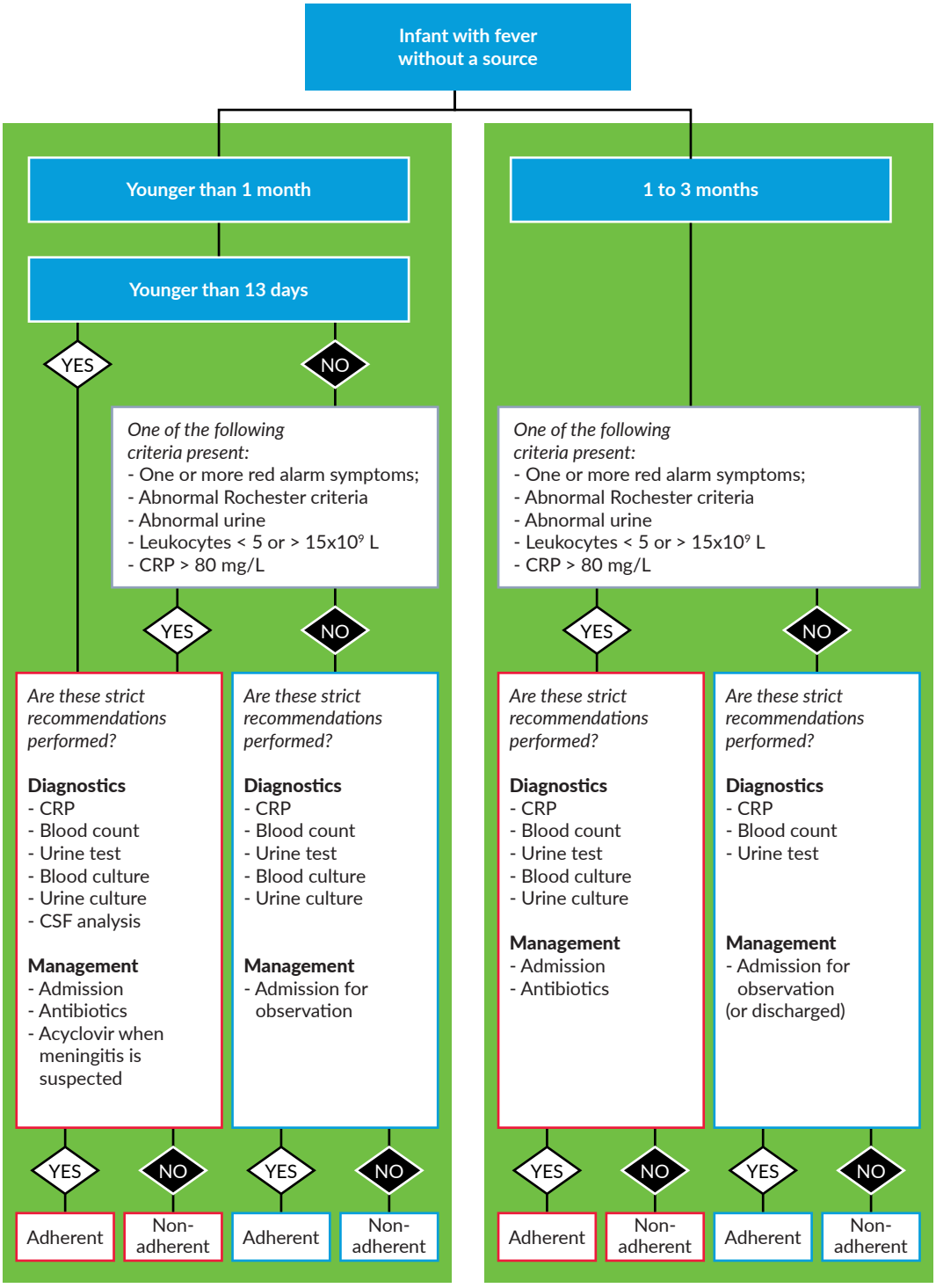
This multicenter retrospective cohort study was performed at the Tergooi hospital in Blaricum and the Noordwest Ziekenhuisgroep in Alkmaar, two non-academic teaching hospitals in the Northern Holland province in the Netherlands. Medical ethical approval for the study was obtained from the Scientific Review Committee of Tergooi hospital in October 2019 (reference number KV19.056, registration number 19.51). Informed consent by pediatric patients' caregivers was not required. The study was not subject to the Medical Research Involving Human Rights Act (WMO), since no interventions were performed, and data were collected retrospectively.[26]

Study population

We retrospectively assessed the medical records of all infants, who were presented at the emergency departments of the two participating hospitals during the period of 30th of September 2017 to 1st of October 2019. Infants were eligible for this study if they were younger than three months of age, were previously healthy, had a temperature of >38.0°C or <36.0°C at home or at the emergency department and, were suspected of having an infection. Infants were excluded if they met criteria for an early onset neonatal sepsis (within 72 hours after birth), suffered from a hospital-acquired fever or post-operative fever, or in case an evident focus was found in line with the clinical presentation.[25] The mentioned definitions, inclusion and exclusion criteria in our study were obtained from the Dutch guidelines to guarantee a valid study population representative for the patient population targeted by the guidelines.

The Dutch FWS Guidelines

The Dutch guidelines developed flowcharts by age categories (infants younger than one month and between one and three months) with criteria to categorize infants into low-, moderate- and high- risk of a serious infection (figure 1).[27-29] Criteria that categorize infants younger than three months into a high risk of infection are presence of one or more red alarm symptoms (as bulging fontanelle or tachypnoea >60/min etc.), abnormal Rochester criteria, abnormal urine, abnormal white blood cell (WBC) count (leukocytes <5 or >15x10^9/L), and c-reactive protein



Legend: ☐ high risk of infection, ☐ low/medium risk of infection, * as described in the traffic light system[18]

(CRP) >80mg/L (figure 1). The Rochester criteria determine whether febrile infants younger than 60 days are at low risk for a serious infection.[25] Depending on the age category and the predefined risk of infection, the guidelines recommend clinicians which diagnostics and management need to be performed.

Data collection

The following data were extracted from the medical records: length of stay in the hospital, duration of antibiotic therapy, started with antibiotic treatment during hospital stay due to deterioration of the infant (>12hr after presentation), readmission (< 5 days), representation (< 5 days), transfer to an intensive care unit or mortality. All potential diagnoses of infants with FWS as mentioned by the guidelines were extracted and defined according to their definitions. A serious bacterial infection was thus defined as a culture confirmed infection that needed interventions including bacteremia, meningitis, pneumonia, urinary tract infection, septic arthritis or osteomyelitis. In accordance with the Dutch guidelines, a herpes simplex virus (HSV) encephalitis and Kawasaki disease were categorized as a serious infection.[25] All viral test results were recorded. We categorized cases as 'unknown' when no diagnosis was found. All the above information was extracted from the electronic patient file using HiX EPD software. Castor Electronic Data Capture version 1.4 (Ciwit B.V., Amsterdam, The Netherlands) was used to process all clinical report forms.

Data analysis

The primary outcome, adherence to the Dutch guidelines, was defined as performing all diagnostics and management as recommended by the guidelines, classified by age group and by risk of infection. When a recommended diagnostic test was attempted but unsuccessful, this was considered as adherence. The guidelines classify infants between one and three months into low- or moderate risk of infection, but since the recommended diagnostics and management are similar, we combined the low and moderate group. For the secondary outcomes, a multivariate regression analysis was performed to determine which clinical criteria to classify for high risk of infection were predictive for adherence to the guidelines as well as to non-adherence. The charts with ambiguities were discussed within the research group.

Statistical analyses

The $\chi 2$ or Fisher's exact test was used to compare categorical variables. Normally distributed continuous variables between the groups were tested by means of an independent samples t test. Not normally distributed continuous variables were analyzed between the groups by means of a Mann-Whitney U test. A logistic regression model was used to identify the independent criteria for infants between thirteen days and three months with a high risk of infection with adherence as dependent factor. For all comparisons, an alpha value of < 0.05 was considered as significant.

RESULTS

Study population

During the study period, 1284 infants were presented at the emergency departments, of whom 231 infants met the inclusion criteria (figure 1, Supplementary material). None of the included infants had hypothermia at home or at presentation. The patient characteristics, performed diagnostics and management are shown in table 1A and B in supplemental digital content, classified by risk of infection and by age category.

Adherence to the guidelines

The overall adherence to the Dutch guidelines was 118/231 (51.1%). The adherence in infants younger than one month and infants between one and three months was 36/84 (42.9%) and 82/147 (55.8%) respectively (figure 1, Supplementary material).

Serious infection

We found that 37/231 infants younger than three months (16.0%) had a (culture) confirmed serious infection, of whom 31/37 (83.8%) had a urinary tract infection, 5/37 (13.5%) bacteremia, and 1/37 (2.7%) meningitis, respectively (table 1, supplemental digital content). None of the included infants were diagnosed with a septic arthritis, osteomyelitis, pneumonia, herpes simplex virus (HSV) encephalitis or Kawasaki disease. The number of serious infections was significantly higher in the adherence group compared to the non-adherence group for infants between one and three months of age with a high risk of infection (p=0.009).

In eight infants younger than three months, five in the adherence groups and three in the non-adherence groups, antibiotic treatment was started later during hospital stay due to clinical deterioration. Of the infants in the adherence groups antibiotic treatment was not indicated after first management according to the guidelines. One infant in this group proved to have a culture confirmed serious infection (bacteremia). Of the other four infants, two were diagnosed with a parecho/enterovirus and in two of them the diagnosis was unknown. Of the three infants in the non-adherence group in whom antibiotic treatment is started later during hospital stay, one infant was diagnosed with a urinary tract infection. This infant was classified as high risk of infection due to positive urine analysis whereas this result was interpreted by clinicians as contaminated. Of the other two infants, one infant had a parecho/enterovirus and in one infant the diagnosis was unknown (table 1, supplemental digital content).

Clinical criteria to classify as high risk of infection as predictors of adherence

Multivariate regression analysis for 105 infants between thirteen days and three months showed four criteria that predicted adherence to the Dutch guidelines for infants with a high risk of infection: presence of one or more red alarm symptoms, abnormal Rochester criteria, abnormal urine and CRP >80 mg/L (table 2). As shown in the univariate regression analysis, of all criteria classifying infants at high risk of infection, the WBC count (leukocytes <5 or >15x10^9/L) presents as an opposite predictive value for adherence (OR 0.4, p-value 0.049) (table 2).

Criteria (n=150)**	**Univariate regression OR (95% CI)**	**Multivariate regression OR (95% CI)**
One or more red alarm symptoms	2,4 (1.0-6.0)	7,7 (2.1-28.0)*
Abnormal Rochester criteria	2,7 (1.0-6.7)*	8,3 (2.2-30.6)*
Abnormal Urine	2,7 (1.1-6.6)*	8,5 (2.3-31.3)*
Leukocytes	0,4 (0.2-1.0)*	-
CRP	6,6 (1.7-26.3)*	12,8 (2.4-70.0)*

Table 2 Clinical criteria to classify as high risk of infection as predictors of adherence

* P < 0.05

** 105 infants between 13 days and three months of age with a high risk of infection

CRP, c-reactive protein.

Non-adherence

The diagnostics and management that were not performed in case of non-adherence were obtaining a urine culture in 76/113 (67.3%), a blood culture in 65/113 (57.5%), or starting intravenous antibiotic treatment in 43/113 (38.1%) of cases (table 3). Non-adherence in performing a urine culture (p=0.006), urine sediment (p=0.016) and antibiotic treatment (p=0.039) differed significantly between different age categories.

Not performed diagnostics and management	Non-adherence group N=113	Non-adherence < 1 month N=48	Non-adherence 1-3 month N=65	P-value
Diagnostics, N (%)				
Blood count	6 (5.3%)	1 (2.1%)	5 (7.7%)	0.239
CRP	5 (4.4%)	0	5 (7.7%)	0.071
Urine sediment	18 (15.9%)	**3 (6.3%)**	**15 (23.1%)**	**0.016**
Urine culture	76 (67.3%)	**39 (81.3%)**	**37 (56.9%)**	**0.006**
Blood culture	65 (57.5%)	25 (52.1.9%)	40 (61.5%)	0.315
CSF analysis	18 (15.9%)	18 (37.5%)	-	- *
Management, N (%)				
Admission	14 (12.4%)	3 (6.3%)	11 (16.9%)	0.089
Parenteral antibiotics	43 (38.1%)	**13 (27.1%)**	**30 (46.2%)**	**0.039**
Acyclovir by meningitis	1 (0.9%)	1 (2.1%)	-	- *
Discharged	-	-	-	

Table 3 Number of not performed diagnostics and management in the non-adherence group classified by age of category.
Bold values denote statistical significance at the P < 0.05 level. * not strictly recommended for infants between 1 and 3 months. CRP, c-reactive protein; CSF, cerebrospinal fluid.

Of the infants in the non-adherence group 13 infants afterwards proved to have a bacterial culture confirmed serious infection. Ten infants had a urinary tract infection and in one infant antibiotic treatment was delayed because of non-adherence. This infant had positive urine analysis that interpreted by clinicians as urine sample contamination. In the other infants with a urinary tract infection less diagnostics were performed than recommended in the guidelines. There were namely no blood cultures and in some cases no cerebrospinal fluid tests performed. Two infants had a bacteremia and one infant had a meningitis in whom antibiotic treatment was started at presentation but less diagnostics were performed (urine cultures or cerebrospinal fluid test) than recommended.

DISCUSSION

The aim of this study was to determine the adherence to the Dutch FWS guidelines and the percentage of serious infections in infants younger than three months of age. We found that adherence to the Dutch guidelines was low. Serious infections occurred more frequently in infants in the adherence groups compared to the non-adherence groups, although no relevant significant differences in clinical outcomes were observed between the groups. In case of non-adherence, mostly a urine or blood culture was not performed, antibiotic treatment was not started, or an abnormal WBC alone was not considered as an indicator for sepsis work-up.

The observed incidence of a culture-confirmed serious infection was approximately 16% which is slightly higher than reported in literature.[2-7] We found that 13.4% were suffering from a urinary tract infection, making up 83.8%

of all serious infections in this study population. Bacteremia and bacterial meningitis in febrile infants were rare occurring in 2.2% and 0.4%, respectively, which is consistent with current literature.[2,14]

In infants aged one to three months with a presumed high risk of infection, bacterial infection rate was significantly lower in the non-adherence group compared to the adherence group (16 versus 44%). If the guidelines were followed in all cases, this could have led to unnecessary testing and treatment. One could hypothesize that the clinician's own judgement on risk of infection has prevented extra unnecessary testing and treatment that were recommended by the guidelines in these cases. It should be noted that the percentage of serious infections in the non-adherence groups may be underestimated because a lower number of bacterial cultures were performed than recommended. However, only one case in this group received delayed antibiotic treatment which could have been prevented in case of guideline adherence. Additionally, one of the explanations for extensive culture testing as recommended by the guidelines, is that a urinary tract infection can potentially lead to meningitis in younger infants. As more recent systematic reviews support, the prevalence of concomitant meningitis in urinary tract infections is very low.[30,31]

An abnormal WBC count only was negatively associated to adherence, meaning that an abnormal WBC count without additional risk factors for infection did not motivate clinicians to perform a full sepsis work-up and to start intravenous antibiotics. This reasoning can be supported by a recent study in 4313 febrile infants demonstrating that WBC count alone was not an accurate predictor of serious invasive infections.[28] This can be explained by the newer more accurate inflammatory markers such as CRP and procalcitonin, and the post pneumococcal conjugate vaccine period.[27,32,33] For example, previously, a higher WBC counts showed a stronger association with Streptococcus Pneumoniae bacteremia compared to other pathogens but since the introduction of the pneumococcal conjugate vaccine this has become less relevant.[27] Our data emphasize this statement, since none of the infants younger than three months of age with an abnormal WBC count without additional risk factors, had a culture confirmed serious infection. Therefore, we recommend that the guidelines remove an abnormal WBC count as indicator for starting intravenous antibiotics and full sepsis work-up.

In case of non-adherence, urine diagnostics were also often not performed despite the guidelines recommendations.[2-7,34] The NICE guidelines advise to perform a urine culture in all infants younger than three months of age independent of the results of the dipstick, because of the higher rates of false negative dipsticks.[25,35-38] Indeed, we also found three infants with false negative dipsticks. This amount is likely to be underestimated because in many infants with a normal dipstick, no urine culture has been performed. Clinicians must be aware of the importance of performing urine cultures because it was a major contributor to non-adherence, whereas urinary tract infections are the most common serious infection in young infants.[25,35-38] Interestingly, entero- and parechovirus diagnostics were often performed. The current guidelines do not strictly recommend these diagnostics although they may be considered. A recent study showed that in children under three years old with a fever without a source, 34.8% had a positive blood enterovirus and 5.9% a positive blood parechovirus PCR.[37] We think the threshold to perform viral diagnostics should be lowered because viral infections relatively common in pediatric patients and identifying viral infections could probably reduce length of admission and time exposed to antibiotics.[37-39]

Moreover, the number of criteria and the possibility to interpret criteria in different ways contribute to non-adherence.[42,43] For example, the guidelines constructed a flowchart for infants younger than one month of age with five different criteria to classify infants as high risk of infection. One of these criteria is the presence of red alarm symptoms described in the traffic light system.[25] According to this traffic light system, being an infant under one month of age is already a red alarm symptom, and consequently all infants under the age of one month are per definition classified as high risk. These potentially confusing interpretations could lead to unintended variability in management of infants with FWS.

We acknowledge that our study had several limitations. We may have missed infants because the clinicians reported a focus for fever, while this focus may not have been consistent with the clinical presentation of that infant. Further we may have included infants who were classified by the clinicians as fever without an apparent source, despite having symptoms at presentation that were not reported in the records. This may have led to selection bias. Moreover, since follow up data from not participating hospitals is lacking this may have led to an underestimated percentage of serious infection and clinical outcomes. As parents were cautiously instructed to present their infants to one of the participating hospitals in case of clinical deterioration, we estimate the number of missed deteriorated infants to be low.

In conclusion, adherence to the Dutch FWS guidelines was low, but there were no significant differences in clinical outcomes between the adherence and the non-adherence group. We advocate that the guidelines need to be adjusted to increase adherence.

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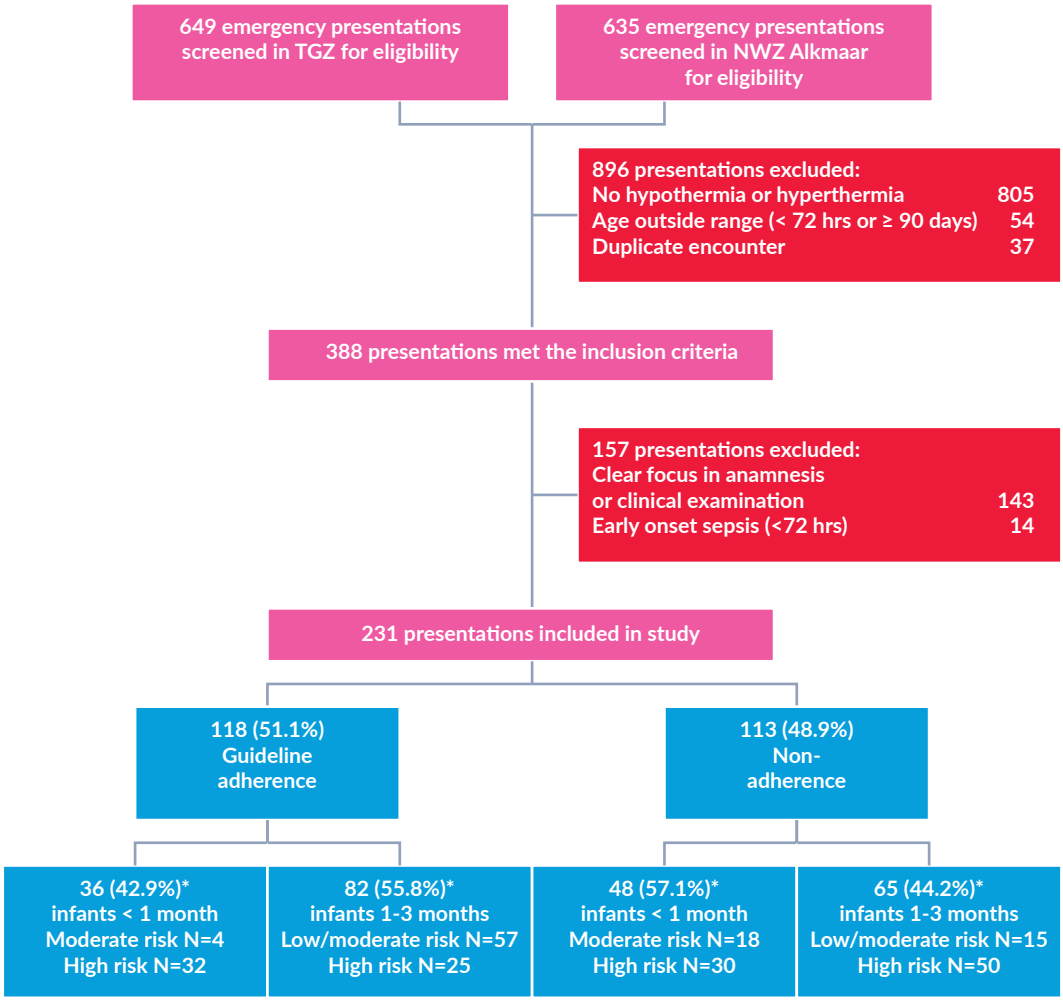
SUPPLEMENTARY MATERIAL



* *Percentages compared to age category*

Supplementary material Figure 1 Study flowchart and adherence to the Dutch guidelines (N (%))

The **supplemental digital content** can be accessed online via https://links.lww.com/INF/E99

Chapter VIII

Prospective multicenter evaluation of adherence to the Dutch guideline for children aged 0 - 16 years with fever without a source

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ABSTRACT

Background

Evaluation of guidelines in current practice is a crucial step in guideline improvement. Retrospective evaluation of the Dutch national guideline for children with fever without an apparent source (FWS) showed 50% adherence in young infants. We aimed to prospectively evaluate adherence to the Dutch guideline and its outcomes in current practice.

Methods

This observational prospective multicenter study included children aged three days to sixteen years old presented for FWS at the emergency department in seven participating secondary and tertiary care hospitals in the Netherlands. Adherence to the Dutch FWS guideline, adapted from the National Institute for Health and Care Excellence (NICE) of the United Kingdom, was evaluated and patterns in non-adherence and the impact of non-adherence on clinical outcomes and resource use were explored.

Results

Full adherence to the guideline was reported in 79/159 (50%). Adherence was lowest in patients younger than one month with 8/30 (27%) compared to 37/64 (58%) in patients older than three months. In patients categorized as high risk of severe infection adherence was 22/71 (31%), whereas adherence in the low risk group was 33/41 (81%). Differences in adherence were significant between age categories ($P = 0.018$) and between risk categories ($P < 0.001$). In case of non-adherence less urinalysis, less bacterial cultures (blood, urine and cerebral spinal fluid) and less empirical antibiotic treatment was performed than recommended by the guideline (all $P < 0.050$). Clinical outcomes were not significantly different between the non-adherence and adherence group, particularly in missed severe infections.

Conclusion

In our multicenter prospective evaluation of the Dutch guideline for children presenting with FWS, the high non-adherence rate of 50% did not lead to unfavorable clinical outcomes. Our results provide clues required for targeted future guideline improvement.

INTRODUCTION

Fever without an apparent source (FWS) is one of the most common reasons for children to visit the emergency department (ED).[1] Most cases of FWS are caused by a mild self-limiting infection, while approximately 6-15% is caused by a severe infection requiring immediate treatment.[2] Clinical presentation is often nonspecific in young children, hampering prompt recognition and adequate management. This diagnostic uncertainty in differentiating severe from self-limiting infections leads to overtesting and overtreatment increasing the burden for children presenting with FWS. Furthermore, this increases health care costs, with an almost five-fold higher use of ED resources reported among infants younger than three months compared to children older than six months.[3] The Dutch Association of Paediatrics (NVK) published the national guideline "Fever in secondary care setting in children aged 0-16 years" in 2013, aiming to improve early recognition of severe infections without increasing unnecessary diagnostic testing.[4] The Dutch guideline, adapted from the National Institute for Health and Care Excellence (NICE) of the United Kingdom, aimed to provide a step-by-step pathway to assess the risk of infection through their traffic light system and subsequently recommends diagnostic testing and treatment.

The purpose of guidelines in general is to ensure consistent and effective evidence-based health care. Practice variation in FWS management, however, is substantial. For example, in the United States the admission and lumbar puncture rates for infants with FWS varied between 40-90% among 26 ED's.[5] A multicenter study reported wide variation in prescriptions of broad-spectrum antibiotics in febrile children between European ED's, of which at least half of the participating ED's had implemented the Dutch or NICE guideline.[6] In a study on the impact of FWS guidelines, the availability of a guideline was not associated with reduced costs and some guidelines even resulted in increased costs without improvement on clinical outcomes.[7] This substantiates the need for guideline evaluation to assess its adherence and applicability in current practice, particularly in the context of considerable practice variation. In turn, this knowledge may reduce unwanted practice variation and ineffective use of guidelines.

Evaluation of guideline adherence and outcomes in current practice is a crucial step in guideline improvement. After implementation of the Dutch guideline our retrospective study was the first evaluation of this guideline, measuring low to moderate adherence in children younger than three months.[8] Multicenter prospective evaluation of adherence to the national guideline, including all age groups, is needed to corroborate these findings. Thus, this prospective multicenter study aimed to evaluate the adherence to diagnostic and treatment recommendations of the Dutch national guideline for children aged 0-16 years with FWS. As our secondary aims, we investigated patterns in non-adherence and the impact of non-adherence on clinical outcomes and on the use of diagnostic and therapeutic resources. Our results aim to provide clues required for targeted future guideline improvement.

METHODS

Study design and participants

This observational prospective multicenter study included children presented for FWS at the ED in one of seven participating secondary and tertiary care hospitals, organized in the Pediatric Research and Evaluation Network (PREN) Amsterdam, in the North-West region of the Netherlands during December 2020 to May 2022. Inclusion criteria, directly adopted from the national guideline, were (I) children aged 3 days to 16 years; (II) presented for FWS, defined as a temperature of ≥ 38.0 °C at home or during ED visit and (IIA) no evident focus of infection after history and physical examination or (IIB) a clinical presentation not fitting the potential focus.[4] Exclusion criteria were: children with hospital-acquired or post-operative fever and typical febrile seizures without a diagnostic work-up for the focus of the fever.

FWS guideline definitions

Severe infection was defined according to the guidelines: a confirmed HSV encephalitis, bacteremia, bacterial meningitis, urinary tract infection, septic arthritis, osteomyelitis, pneumonia or Kawasaki disease.[4] The guideline

recommends separate diagnostic and treatment pathways based on age category of the patient and the risk of severe infection category. Age categories are defined as children younger than one month, one to three months, and older than three months and risk of severe infection is categorized as low (green), intermediate (amber) and high risk (red) (figure 1). The risk of severe infection is categorized based on the combination of age, red or amber flags of the NICE traffic light system (supplementary table 1), Rochester criteria for children younger than two months (supplementary table 2) and the results of primary diagnostic testing. Primary diagnostic testing include dipstick urinalysis, C-reactive protein (CRP) and, in children younger than three months, white blood cell count (WBC). Subsequently, patients younger than one month can be categorized as having an intermediate or high risk of severe infection while patients aged one to three months or older than three months can be categorized as having a low, intermediate or high risk of severe infection (figure 1). Depending on the age category and risk of infection category, the guideline provides recommendations for secondary diagnostic testing, hospital admission and empirical antimicrobial treatment similar to the NICE guideline (figure 1). Secondary diagnostic testing include: blood culture, urine culture, cerebral spinal fluid (CSF) culture and analysis, feces culture, Polymerase Chain Reaction (PCR) testing of CSF, serum, throat swab and feces samples for Herpes Simplex virus (HSV), entero- and parechovirus, influenzavirus and respiratory viruses including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and chest X-rays. Treatment recommendations include empirical intravenous (IV) antibiotics, empirical oral antibiotics and empirical IV acyclovir. When rapid viral testing was positive for influenza or respiratory syncytial virus (RSV) in its endemic season, the guideline states to only perform a diagnostic work-up for a potential severe infection in case of an ill-appearing patient. In case of a positive rapid viral test and a well-appearing patient, bacterial cultures and empirical antibiotic treatment are not indicated.

Age < 1 month

1 or more criteria?

age < 13 days, 1 or more red flags, abnormal Rochester criteria, abnormal urinalysis, leukocytes < 5 or > 15x10^{9}L, CRP > 80 mg/L

YES

Diagnostics

- blood count
- CRP
- blood culture
- urinalysis
- urine culture
- CSF analysis
- CSF culture
- CSF PCR for HSV entero-/parechovirus

Treatment

- admission
- IV antibiotics
- IV acyclovir in case of suspected meningitis

NO

Diagnostics

- blood count
- CRP
- blood culture
- urinalysis
- urine culture

Treatment

- admission for observation

Age 1 to 3 months

1 or more criteria?

1 or more red flags, abnormal Rochester criteria*, abnormal urinalysis, leukocytes < 5 or > 15x10^{9}L, CRP > 80 mg/L

YES

Diagnostics

- blood count
- CRP
- blood culture
- urinalysis
- urine culture

Treatment

- admission
- IV antibiotics

NO

2 or more orange flags OR 1 orange flag AND CRP > 20 mg/L

YES

Diagnostics

- blood count
- CRP
- urinalysis

Treatment

- admission for observation

NO

Diagnostics

- blood count
- CRP
- urinalysis

Treatment

- admission or
- discharge

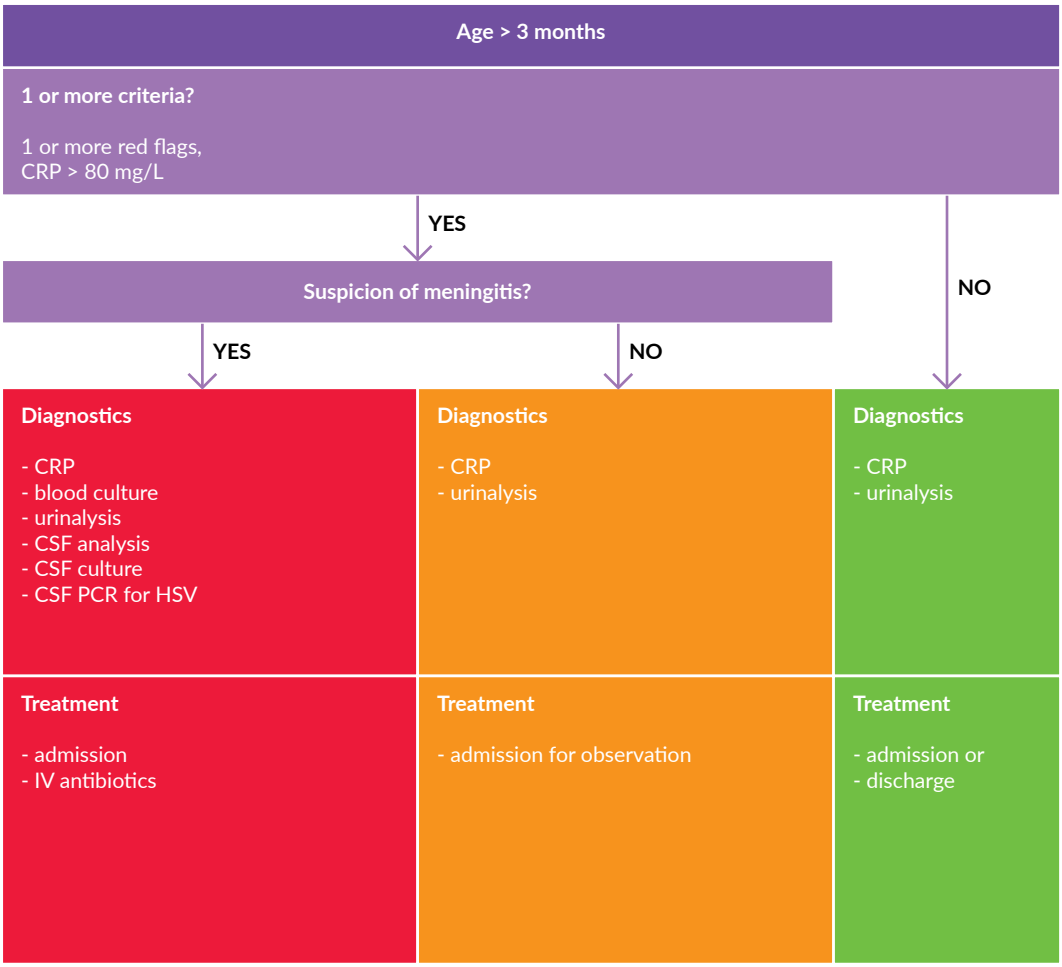

Figure 1 The Dutch national FWS guideline recommendations
Pathway for diagnostic and treatment recommendations per age category as defined by the Dutch FWS guideline and derived from the NICE traffic light system. * Rochester criteria only applicable in children aged younger than two months. Abbreviations: CSF, cerebral spinal fluid; CRP, C-reactive protein; HSV, herpes simplex virus; IV, intravenous; PCR, polymerase chain reaction assay.

Data collection

Eligible patients were managed in the ED according to the judgement of the treating physician. After informed consent was obtained, data regarding the ED visit evaluation and management was collected prospectively by the treating physician. ED visit data included patient characteristics, history and physical examination during ED visit, diagnostic testing and treatment, testing results and clinical outcomes collected seven days after the initial ED visit. Subsequently, two independent researchers assessed adherence of each case to the Dutch FWS guideline.

Data analysis and outcomes

The primary study outcome was the percentage of cases with full adherence to all the recommendations of the guideline. Adherence was defined as: 1) full adherence to all recommendations, 2) non-adherence, subdivided in non-adherence to diagnostic and/or treatment recommendations. Cases with an unclear adherence or diagnosis were discussed in the study team blinded for the hospital and treating physician. Antibiotic treatment performed while this was not recommended was also considered non-adherence. In case CRP or urinalysis was missing and risk category could not be determined, the risk category was considered as missing data.

For the secondary study outcomes the adherence group and the non-adherence group were compared in terms of: patient characteristics, clinical outcomes and the use of diagnostic and therapeutic resources. For patterns in non-adherence, the patient characteristics were compared between adherence groups. For the clinical outcomes we assessed potentially missed severe infections in the adherence and non-adherence group based on reported delayed antibiotic treatment (>12 hours) in confirmed bacterial infections, ED revisits and (re)admissions within 7 days after initial visit. Further clinical outcomes included: final discharge diagnosis as reported in medical charts, need for IV fluids, O2 support or ICU transfer, mortality, length of admission, delayed antibiotic treatment (>12 hours) overall. The use of resources was measured as the number of performed testing and treatment per age and risk category in the adherence and the non-adherence group.

Since rapid diagnostic testing of SARS-CoV-2 was implemented in the course of the study and recommendations for this novel virus were not yet described in the FWS guideline, we excluded patients with a positive SARS-CoV-2 rapid test from the FWS cohort analysis to be explored separately in the Coronavirus disease 2019 (COVID-19) cohort. Similar to the recommendations for influenza or RSV rapid testing, a positive rapid test for SARS-CoV-2 in the ED was considered a plausible diagnosis in a patient without red flags. Only an ill-appearing patient with a positive rapid test for SARS-COV-2 required a diagnostic work-up for a potential severe infection, according to the FWS guidelines. If SARS-CoV-2 was tested with a regular non-rapid PCR test and results therefore did not impact ED visit management, this patient was included in the FWS cohort. If rapid viral testing was positive for RSV or influenza, these patients were included in the FWS cohort.

Statistical methods

SPSS Statistics version 26.0 (IBM Corp, New York, USA) was used for all analyses. For continuous variables means with standard deviations or medians with interquartile ranges were calculated. Differences between groups in not-normally distributed variables were analyzed with a Mann-Whitney U test. Categorical variables were depicted in proportions and differences between proportions were analyzed using Pearson's chi-square test or Fisher's exact test. The following potential predictors of non-adherence were identified: age category, risk of severe infection category and comorbidity. All variables with clinical importance and/or a $P < 0.250$ in univariable regression analysis were included in the multivariable regression model. For all comparisons an alpha value of < 0.050 was considered statistically significant.

Study approval

The study protocol was approved by the Medical Ethics Committee of the Amsterdam University Medical Centers (W20_309 # 20.344) and a waiver for the Medical Research Involving Human Subjects Act was provided. Written informed consent was obtained from parents/guardians and/or from children above the legal age of consent.

RESULTS

Patient selection

A total of 186 patients was recruited, of whom 159/186 (86%) were included in the FWS cohort and 22/186 (12%) were included in the COVID-19 cohort due to positive rapid diagnostic testing for SARS-CoV-2. Rapid viral testing (performed for SARS-CoV-2, RSV and/or influenza) confirmed a viral infection directly during the ED visit in 30/189 (16%) of all recruited children. A final diagnosis of severe infection was confirmed in 28/186 (15%) while a viral infection was confirmed in 73/186 (39%).

Patient characteristics of the FWS cohort

Characteristics, number of performed diagnostic testing and treatment, final diagnoses and clinical outcomes are shown in table 1 for the FWS cohort and per age category of the FWS cohort. Of the FWS cohort, 30/159 (19%) patients were younger than one month, 65/159 (41%) were one to three months old and 64/159 (40%) were older than three months. The risk of severe infection was categorized as low in 41/159 (26%), intermediate in 37/159 (23%) and high in 71/159 (45%) of the patients while in 10/159 (6%) patients the risk could not be categorized due to missing data. The hospital admission rate was 107/159 (67%) and overall antibiotic treatment rate was 56/159 (34%), of which was given intravenously in 41/159 (26%). Of the patients categorized as high risk of severe infection, 20/71 (28%) were tested for HSV and 13/71 (18%) patients were empirically treated with IV acyclovir. Regarding clinical outcomes, the overall rate of delayed antibiotic treatment in confirmed bacterial infection was 3/159 (2%) and ED revisit with admission was reported in 1/159 (1%). There were no ICU transfers or deaths in the cohort.

Severe infections

A final diagnosis of severe infection was confirmed in 28/159 (18%) of the FWS cohort (table 1). A viral infection was confirmed in 51/159 (32%), while in 81/159 (51%) no pathogen was identified. One patient was diagnosed with a viral and bacterial coinfection. The bacterial infection rate was higher in children categorized as high risk of severe infection compared to low risk according to the traffic light system: 1/41 (2%) in the low risk group compared to 5/37 (14%) in the intermediate and 21/71 (30%) in the high risk group. Similarly, the bacterial infection rate was higher in patients younger than one month compared to the older age categories (table 1).

	FWS cohort	**Age < 1 month**	**Age 1 to 3 months**	**Age > 3 months**
Number	159	30	65	64
Patient history				
Sex, female	66 (42%)	11 (37%)	32 (49%)	23 (36%)
Risk of severe infection				
Low	41 (28%)	-	17 (27%)	24 (44%)
Intermediate	37 (25%)	3 (10%)	11 (17%)	23 (42%)
High	71 (48%)	27 (90%)	36 (56%)	8 (15%)
Comorbidity	10 (6%)	0	1 (2%)	9 (14%)
Vaccination status				
According to program	73 (46%)	-	-	52 (81%)
Age before start program	61 (40%)	30 (100%)	65 (100%)	41 (64%)
Not according to program	1 (1%)	-	-	1 (2%)
Time of ED visit				
06:00 – 12:00h	24 (15%)	8 (27%)	8 (12%)	8 (13%)
12:00 – 18:00h	57 (36%)	8 (27%)	19 (29%)	30 (47%)
18:00 – 00:00h	53 (33%)	8 (27%)	22 (34%)	23 (36%)
00:00 – 06:00h	25 (16%)	6 (20%)	16 (25%)	3 (5%)

Physical examination				
Temperature, mean SD	38.6 C° (0.9)	38.3 C° (0.6)	38.3 C° (0.7)	38.9 C° (1.1)
Respiratory rate, mean SD	38 bpm (13)	45 bpm (13)	40 bpm (12)	33 bpm (13)
Heart rate, mean SD	161 bpm(24)	166 bpm (22)	167 bpm (22)	154 bpm (25)
Capillary refill > 2 sec	27 (18%)	8 (28%)	9 (14%)	10 (17%)
Performed diagnostics				
WBC	143 (90%)	30 (100%)	64 (99%)	49 (77%)
CRP	148 (93%)	30 (100%)	64 (99%)	54 (85%)
Urinalysis	145 (91%)	28 (93%)	60 (92%)	57 (89%)
Blood culture	60 (38%)	23 (77%)	18 (28%)	19 (30%)
Urine culture	73 (46%)	21 (70%)	30 (46%)	22 (34%)
CSF				
- cells/protein/glucose	23 (15%)	12 (40%)	4 (6%)	7 (11%)
- culture	26 (16%)	15 (50%)	4 (6%)	7 (11%)
- PCR HSV	21 (13%)	13 (43%)	4 (6%)	4 (6%)
- PCR entero/parechovirus	21 (13%)	13 (43%)	4 (6%)	4 (6%)
Feces				
- culture	8 (5%)	5 (17%)	1 (2%)	2 (3%)
- PCR entero/parechovirus	38 (24%)	14 (47%)	15 (23%)	9 (14%)
Throat swab				
- PCR entero/parechovirus	16 (10%)	7 (23%)	6 (9%)	3 (5%)
- PCR respiratory viruses	83 (52%)	12 (40%)	41 (63%)	30 (47%)
- PCR SARS-CoV-2	122 (77%)	26 (87%)	51 (79%)	45 (70%)
Chest X-ray	14 (9%)	2 (7%)	0	12 (19%)
Treatment, N (%)				
Admission	107 (67%)	29 (97%)	48 (74%)	30 (47%)
Oral antibiotics	15 (9%)	0	4 (6%)	11 (17%)
IV antibiotics	41 (26%)	21 (70%)	9 (14%)	11 (17%)
IV acyclovir	13 (8%)	9 (30%)	2 (3%)	2 (3%)
Discharge and re-evaluation	13 (8%)	0	1 (2%)	12 (19%)
Confirmed diagnosis				
Bacterial	27 (17%)	11 (37%)	10 (15%)	6 (9%)
Viral	50 (31%)	16 (53%)	18 (28%)	16 (25%)
Bacterial and viral	1 (1%)	0	0	1 (2%)
Unconfirmed	81 (51%)	3 (10%)	37 (57%)	41 (64%)
Severe infection				
Bacteremia	4 (3%)	3 (10%)	0	1 (2%)
Meningitis	2 (1%)	0	2 (3%)	0
UTI	23 (15%)	9 (30%)	8 (12%)	6 (9%)
Septic arthritis	0	0	0	0
Osteomyelitis	0	0	0	0
Pneumonia	4 (3%)	0	0	4 (6%)
HSV encephalitis	0	0	0	0
Kawasaki disease	0	0	0	0

No severe infection				
Enterovirus	18 (11%)	13 (43%)	5 (8%)	0
Parechovirus	4 (3%)	1 (3%)	2 (3%)	1 (2%)
Influenza	6 (4%)	0	4 (6%)	2 (3%)
RSV	2 (1%)	1 (3%)	0	1 (1%)
Rhinovirus	12 (8%)	2 (7%)	5 (8%)	5 (8%)
Other viral	8 (5%)	0	3 (5%)	5 (8%)
Clinical outcomes				
IV fluids	5 (3%)	1 (3%)	1 (2%)	3 (5%)
Respiratory support	2 (1%)	1 (3%)	0	1 (2%)
ICU transfer	0	0	0	0
Mortality	0	0	0	0
Delayed antibiotics	11 (7%)	2 (7%)	5 (8%)	4 (6%)
Delayed antibiotics in confirmed bacterial infection	3 (2%)	0	3 (5%)	0
ED revisit	5 (3%)	0	2 (3%)	3 (5%)
Readmission	1 (1%)	0	1 (2%)	0

Table 1 Patient characteristics of the total FWS cohort and per age category
Abbreviations: - , not applicable; bpm, beats/breaths per minute; C° , Celsius; CSF, cerebral spinal fluid; CRP, C-reactive protein; ED, emergency department; FWS, fever without a source; HSV, herpes simplex virus; ICU, intensive care unit; IV, intravenous; N, number; PCR, polymerase chain reaction assay; RSV, respiratory syncytial virus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2;SD, standard deviation; sec, seconds; UTI, urinary tract infection; WBC, white blood cell count.

Adherence in FWS cohort (n=159)

Full adherence to all recommendations was reported in 79/159 (50%) and non-adherence to at least one recommendation in 80/159 (50%, figure 2). Non-adherence was mostly reported for diagnostic recommendations (68/159, 43%). Non-adherence to one recommendation was 41/159 (26%) while non-adherence to two or three separate recommendations was 24/159 (15%) and to four or more recommendations was 15/159 (9%). Specifically, in case of non-adherence mostly blood or urine cultures and lumbar punctures were not performed (figure 3). Non-adherence to treatment recommendations was higher for starting IV antibiotic treatment than for hospital admission (figure 3). Moreover, 6/71 (9%) of the high risk patients were discharged after the ED visit against guideline recommendation. Of all children aged one to three months with a high risk of severe infection, 65% did not receive IV antibiotics while this was recommended by the FWS guideline. IV antibiotic treatment was started while not recommended by the guideline in 4% of the total cohort.

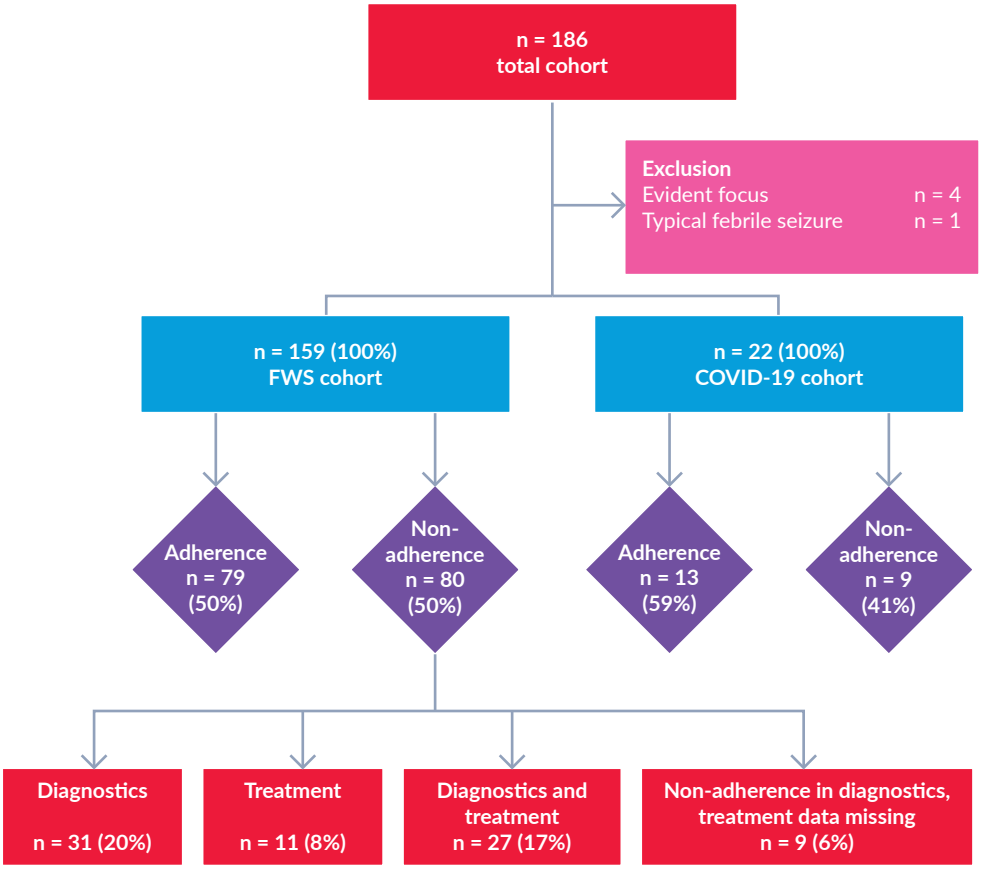

Figure 2 Patient inclusion and adherence

Abbreviations: COVID-19, coronavirus disease 2019; FWS, fever without a source; n, number.

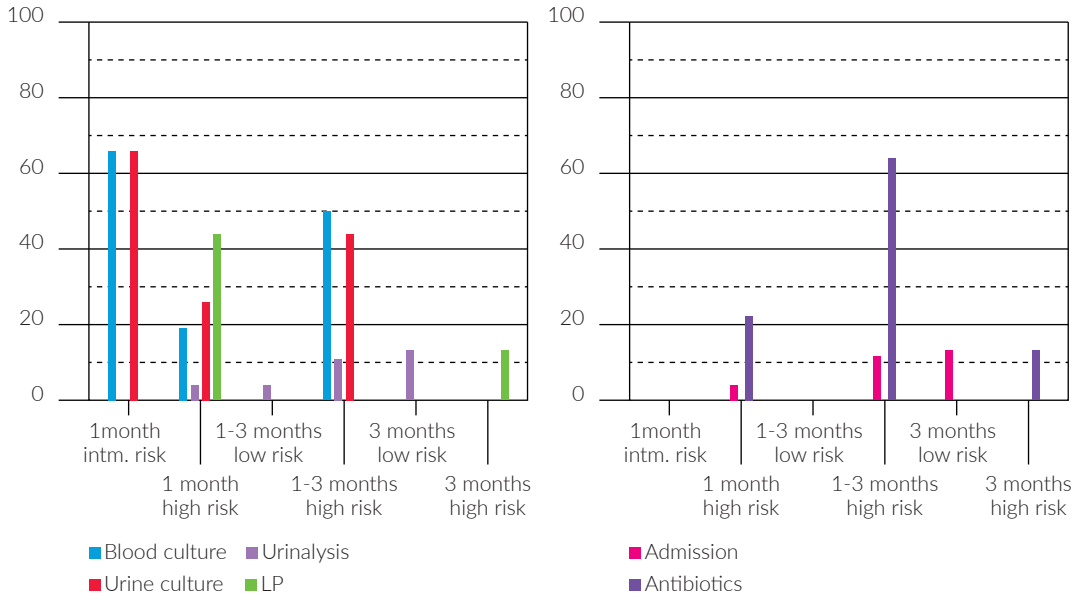

Figure 3 Non-adherence to diagnostic and treatment recommendations
Proportions of not performed recommendations for diagnostic testing and treatment, depicted as percentage of the total subgroup. Abbreviations: 1m, age younger than one month; 1-3m, age one to three months; 3m, age older than three months; intm, intermediate; LP, lumbar puncture.

Patterns of non-adherence

For our secondary aim we evaluated patterns in non-adherence by describing patient characteristics per adherence group. Adherence was lowest in patients younger than one month with 8/30 (27%) compared to 37/64 (58%) in patients older than three months. In patients categorized as high risk of severe infection adherence was 22/71 (31%), whereas adherence in the low risk group was 33/41 (81%). Differences in adherence were significant between age categories ($P = 0.018$) and between risk categories ($P < 0.001$). Figure 4 shows adherence per age and risk category. In multivariable logistic regression (including age category, risk of severe infection and comorbidity) only the risk category was an independent predictor for non-adherence: the high risk category showed an adjusted odds ratio of 8.33 ($P < 0.001$) compared to the low risk category. Besides a lower median age and a higher risk category in the non-adherence as compared to adherence group, there were no significant differences between the adherence and the non-adherence group in the number of severe infections or time of ED visit (table 2).

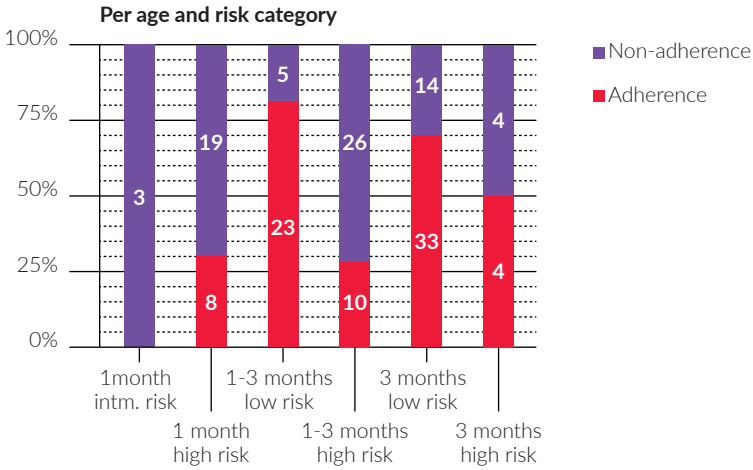

Figure 4 Adherence per age and risk category

Adherence, depicted in the red bars, is shown per age and per risk category in percentages of the total subgroup. Numbers in the bars depict the absolute number of patients within each subgroup.

	Adherence group	Non-adherence group	P
Patient characteristics			
Median age	72 days (IQR 46 – 365)	61 days (IQR 27 – 320)	0.029
Time of ED visit			
06:00 – 12:00h	8/78 (10%)	16/81 (20%)	NS
12:00 – 18:00h	30/78 (39%)	27/81 (33%)	
18:00 – 00:00h	28/78 (36%)	25/81 (31%)	
00:00 – 06:00h	12/78 (15%)	13/81 (16%)	
Severe infection risk			
Low risk	33/78 (42%)	12/81 (15%)	< 0.001
Intermediate risk	23/78 (30%)	17/81 (21%)	
High risk	22/78 (28%)	45/81 (55%)	
Unknown	0	10/81 (12%)	
Confirmed diagnosis			
Bacterial	14/78 (18%)	14/81 (17%)	NS
Viral	22/78 (28%)	29/81 (36%)	NS
Unknown	43/78 (55%)	38/81 (47%)	NS
Clinical outcomes			
Median admission duration	2 days (IQR 0 – 3)	1 day (IQR 0 – 3)	NS
Readmission	0	1/81 (1%)	NS
Delayed antibiotics	5/78 (6%)	6/81 (7%)	NS
Missed severe infections	1/78 (1%)	2/81 (3%)	NS
ICU transfer	0	0	NS
Mortality	0	0	NS

Table 2 Characteristics and clinical outcomes in the adherence and non-adherence group
Abbreviations: ED, emergency department; ICU, intensive care unit; IQR, interquartile range; NS, not significant.

Impact of non-adherence

To evaluate the impact of non-adherence, we compared the clinical outcomes (table 2) and the number of performed diagnostic testing and treatment in the adherence group versus the non-adherence group (table 3 and 4). There were no significant differences in admission rates, admission duration, readmission rates, or missed severe infections between the adherence and non-adherence group (table 2). Regarding missed severe infections, the FWS guideline did not recommend antibiotic treatment in one patient in the adherence group, which was later diagnosed with a bacterial meningitis. In the non-adherence group two patients did not receive antibiotic treatment while this was recommended. These patients received delayed antibiotic treatment and were later diagnosed with a urinary tract infection and a bacterial and viral meningitis, respectively. In the high risk category, there were significantly lower rates of blood/urine cultures, lumbar punctures and antimicrobial treatment in the non-adherence group (table 3). Of the FWS cohort, 30/159 (19%) patients did not receive antibiotic treatment while this was recommended by the guideline. In the low risk category, significantly less urinalysis were performed (table 4).

Risk category	**High risk for severe infection**		
Age category	< 1 month		
Characteristics	Adherence	Non-adherence	p-value
	N = 8	N = 19	
Diagnostics, N (%)			
Blood count	8 (100%)	19 (100%)	-
CRP	8 (100%)	19 (100%)	-
Urinalysis	8 (100%)	17 (90%)	0.567
Blood culture	8 (100%)	14 (74%)	0.280
Urine culture	8 (100%)	12 (63%)	0.068
CSF culture	**8 (100%)**	**7 (37%)**	**0.003**
CSF PCR entero/parechovirus	**7 (88%)**	**6 (32%)**	**0.013**
Treatment, N (%)			
Admission	8 (100%)	18 (95%)	1.00
Antibiotics	8 (100%)	13 (68%)	0.072
Acyclovir	5 (63%)	4 (21%)	0.072
Discharged	0	1 (5%)	1.000

Table 3 Differences in management for high risk of severe infection
For all children categorized as high risk of infection (n = 70) the performed diagnostic testing and treatment are depicted per age group and compared between the adherence and non-adherence group. Proportions were com-

Risk category	**Low/intermediate risk for severe infection**		
Age category	< 1 month		
Characteristics	Adherence	Non-adherence	p-value
	N = 0	N = 3	
Diagnostics, N (%)			
Blood count	0 (0%)	3 (100%)	-
CRP	0 (0%)	3 (100%)	-
Urinalysis	0 (0%)	3 (100%)	-
Blood culture	0 (0%)	1 (33%)	-
Urine culture	0 (0%)	1 (33%)	-
CSF culture	0 (0%)	0 (0%)	-
CSF PCR entero/parechovirus	0 (0%)	0 (0%)	-
Treatment , N (%)			
Admission	0 (0%)	3 (100%)	-
Antibiotics	0 (0%)	0 (0%)	-
Acyclovir	0 (0%)	0 (0%)	-
Discharged	0 (0%)	0 (0%)	-

Table 4 Differences in management for low/intermediate risk of severe infection
For all children categorized as low or intermediate risk of infection (n = 72) the performed diagnostic testing and treatment are depicted per age group and compared between the adherence and non-adherence group. Propor-

1 to 3 months Adherence N = 7	Non-adherence N = 28	p-value	>3 months Adherence N = 4	Non-adherence N = 4	p-value
7 (100%)	28 (100%)	-	4 (100%)	4 (100%)	-
7 (100%)	28 (100%)	-	4 (100%)	4 (100%)	-
7 (100%)	25 (89%)	0.365	4 (100%)	4 (100%)	-
7 (100%)	**8 (27%)**	**0.001**	4 (100%)	4 (100%)	-
7 (100%)	**11 (39%)**	**0.008**	4 (100%)	2 (50%)	0.429
3 (43%)	**0 (0%)**	**0.005**	4 (100%)	3 (75%)	1.000
3 (43%)	**0 (0%)**	**0.005**	3 (75%)	1 (23%)	0.486
7 (100%)	24 (86%)	0.288	4 (100%)	4 (100%)	-
7 (100%)	**5 (18%)**	**0.001**	4 (100%)	3 (75%)	1.00
2 (29%)	**0 (0%)**	**0.035**	2 (50%)	0 (0%)	0.429
0 (0%)	4 (14%)	0.562	0 (0%)	0 (0%)	-

pared between the adherence and non-adherence group with chi-square or Fisher's exact testing. An alpha value of < 0.050 was considered statistically significant and depicted in bold. Abbreviations: CRP, C-reactive protein; N, number; PCR, polymerase chain reaction.

1 to 3 months Adherence N = 22	Non-adherence N = 3	p-value	>3 months Adherence N = 32	Non-adherence N = 12	p-value
22 (100%)	3 (100%)	-	29 (91%)	10 (83%)	0.497
22 (100%)	3 (100%)	-	32 (100%)	11 (92%)	0.273
22 (100%)	3 (100%)	-	**31 (97%)**	**9 (75%)**	**0.025**
1 (5%)	1 (33%)	0.230	7 (22%)	2 (17%)	1.000
9 (41%)	1 (33%)	1.000	13 (41%)	2 (17%)	0.171
0 (0%)	1 (33%)	0.120	0 (0%)	0 (0%)	-
0 (0%)	1 (33%)	0.120	0 (0%)	0 (0%)	-
14 (64%)	1 (33%)	0.543	17 (53%)	3 (25%)	0.095
0 (0%)	1 (33%)	0.120	7 (22%)	6 (50%)	0.069
0 (0%)	0 (0%)	-	0 (0%)	0 (0%)	-
8 (36%)	2 (67%)	0.543	15 (47%)	9 (75%)	0.173

tions were compared between the adherence and non-adherence group with chi-square or Fisher's exact testing. An alpha value of < 0.050 was considered statistically significant and depicted in bold. Abbreviations: CRP, C-reactive protein; N, number; PCR, polymerase chain reaction.

Adherence in COVID-19 cohort

In 11/22 (50%) of the COVID-19 cohort there was no ill-appearance reported and the other 11/22 (50%) were reported with an ill-appearance, meaning the presence of a red flag or aged younger than one month. All the well-appearing patients did not receive bacterial cultures and empirical antibiotic treatment after their rapid test diagnosis and were, thus, managed according to the guideline. Regarding the ill-appearing patients, there was adherence to the guideline in 2/11 (18%) of patients, meaning a total adherence to the Dutch FWS guideline of 13/22 (59%) in the COVID-19 cohort. Hospital admission rate was 8/22 (36%) and IV antibiotic treatment was 2/22 (9%). One patient with COVID-19 also reported having a co-infection with influenza, while there were no bacterial coinfections in this cohort.

DISCUSSION

We found adherence to the Dutch national guideline in half of children presenting with FWS at the ED. Adherence to the guideline was lower in children younger than one month compared to older than three months and lowest in children categorized as high risk of severe infection. In the non-adherence group significantly less urinalysis, bacterial cultures, lumbar punctures and antimicrobial treatment were performed compared to the adherence group with no differences in missed severe infections. Moreover, rapid viral diagnostic testing for SARS-CoV-2, RSV or influenza identified a viral focus in 16%. Our findings on patterns and impact of non-adherence identify opportunities for future guideline improvement to optimize emergency care in children presenting with FWS.
We were able to corroborate the findings from our retrospective study in infants younger than three months in this multicenter prospective study covering all age groups.[8] Non-adherence was particularly high in the youngest age or the high risk groups for which the guideline provides numerous recommendations compared to the older or low risk children. With regard to evaluation of the NICE guideline, an upcoming publication of a European consortium similarly showed non-adherence across emergency departments and specifically regarding measurement of NICE-recommended vital signs another study reported 52% non-adherence.[9, 10] Non-adherence can be explained by several factors concerning the physician's knowledge, attitudes and behavior as well as factors concerning the guideline itself.[11] We deliberately did not include a survey of reasons for non-adherence as to not affect behavior of physicians during our adherence evaluation. Some patterns of non-adherence could indicate a lack of physician's awareness which should be targeted for education to improve adherence. For instance, less urinalysis and urine cultures were performed than recommended by the guidelines. Moreover, often urine cultures were not performed after a negative urinalysis while urinalysis of young infants does not have 100% rule-out value for a urinary tract infection.[12] As urinary tract infections are the most frequent cause of FWS yet their clinical presentation remarkably nonspecific, this may require specific attention.[13]

The reported patterns of non-adherence did, however, identify specific recommendations which could be reevaluated and improved. Physicians did not start antibiotic treatment in the majority of children aged one to three months categorized as high risk, indicating that physicians applied a higher threshold to antibiotic treatment than the guideline. Presence of one red flag already categorizes as high risk, while in a validation study of the traffic light system most red flags showed little individual rule-in value for severe infection across multiple datasets.[14] A large meta-analysis also calculated roughly half the rate of severe infection in this age group compared to younger infants.[15] As there was no increase of missed severe infections in our cohort this may indeed indicate opportunities to critically reevaluate the indication for bacterial cultures and treatment in this group. Furthermore, the step-by-step approach of the Dutch guideline can require venous puncture for primary as well as for secondary diagnostic testing. To assess the need for secondary testing such as bacterial blood cultures, the values for WBC or CRP are necessary which would require a second venous puncture. This undesirably increases the burden of children evaluated for FWS.

Rapid viral diagnostic testing, including SARS-CoV-2, RSV and influenza, revealed a plausible source in 16% of all FWS cases. Although these tests may mostly be of value during their endemic seasons, this illustrates the potential to decrease further bacterial testing and treatment. In line with the absence of bacterial coinfections in our cohort,

others studies showed a significantly lower risk for severe infection in febrile infants positive for viral infections compared with virus-negative infants.[16, 17] In the study of Mahajan et al. the risk in virus-negative infants was still considered non-negligible.[16] Criteria for their study participation included having a blood culture performed, implicating that these cohorts may have had a higher a-priori risk for severe infection than our targeted population of all children presenting with FWS. As viral PCR testing for enterovirus, although not available as rapid test, has also shown potential to shorten admission duration and use of antibiotic treatment, evidence-based guidance on the use of viral testing (both rapid and non-rapid methods) should be implemented in the revised FWS guideline.[18]

This guideline evaluation study faced several limitations. First, we were not able to register patients presented at the ED that were not recruited by the physician or refused participation. Selection bias could therefore have overestimated the number of severe patients, as the physician may have not considered using the guideline in very well-appearing FWS patients. Second, adherence could be overestimated if participation to the study influenced the physician's behavior. As our primary and secondary outcomes are very comparable to our previous adherence study, which is less vulnerable to these types of bias due to its retrospective design, the impact on outcomes may be negligible. Third, our inclusion partly took place during the COVID-19 pandemic, which could have influenced the epidemiology of other pathogens and health care seeking behavior.

Concluding, in our multicenter prospective evaluation of the Dutch guideline for children presenting with FWS the high non-adherence rate did not lead to unfavorable clinical outcomes. In case of non-adherence physicians have used less ED resources than the guideline recommended without increasing missed severe infections. This indicates opportunities to adjust the guidelines and, in particular, to implement evidence-based guidance on viral testing in children with FWS.

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SUPPLEMENTARY MATERIAL

	Low risk (green)	Intermediate risk (amber)	High risk (red)
Color	Normal color	Pallor reported by parent/carer	Pale/mottled/ashen
Activity	Responds normally to social cues Content/smiles Stays awake or awakens quickly Strong normal cry or not crying	Not responding normally to social cues No smile Wakes only with prolonged stimulation Decreased activity Disease course different according to parent/carer Ill-appearance according to physician	No response to social cues Does not wake or if roused does not stay awake Weak, high-pitched or continuous cry
Respiratory	Normal breathing	Nasal flaring Tachypnoea: RR > 50 bpm for 6 – 12 months RR > 40 bpm for > 12 months Oxygen saturation ≤ 95% in air Crackles in the chest	Grunting Tachypnoea: RR > 60 bpm Moderate or severe chest indrawing Decreased breathing sounds
Circulation	Normal skin and eyes Moist mucous membranes	Tachycardia: > 160 beats/minute for < 1 yr > 150 beats/minute for 1–2 yrs > 140 beats/minute for 2–5 yrs CRT ≥ 3 seconds Dry mucous membranes Poor feeding in infants Reduced urine output	Reduced skin turgor
Other	None of the amber or red symptoms or signs	Fever for > 5 days Rigors Swelling of a limb or joint Non-weight bearing limb or not using an extremity	Non-blanching rash Bulging fontanelle Neck stiffness Status epilepticus Focal neurological signs Focal seizures

Supplementary table 1 Green, amber and red flags indicating risk for severe infection
Traffic light system with clinical signs and symptoms indicating risk for severe infection as defined by the Dutch FWS guideline, derived from the NICE guideline. Abbreviations: bpm, breaths per minute; CRT, capillary refill time; RR, respiratory rate; yr, year.

Clinical criteria	born at term (≥ 37 weeks gestation)
	no treatment for unexplained hyperbilirubinemia
	no previous antimicrobial therapy
	no chronic or underlying illness
	no previous hospitalization
	well appearing (regarding feeding, activity, alertness, tone, peripheral circulation, breathing)
	no evidence of skin, soft-tissue, bone, joint, or ear infection
Laboratory criteria	white blood cell count of 5 – 15 × 10^9/L
	absolute band count of < 1,5 × 10^9/L
	platelet count of > 150 × 10^9/L
	urinalysis white blood cells <10 per field
	urine leukocyte esterase negative
	urine nitrate negative

Supplementary table 2 Rochester criteria for low risk of severe infection
Rochester criteria as defined by the Dutch FWS guideline.

Chapter IX

Fever without a source in children: international comparison of guidelines

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ABSTRACT

Background

Fever without a source (FWS) in children poses a diagnostic challenge. To distinguish a self-limiting infection from a serious infection, multiple guidelines have been developed to aid physicians in the management of FWS. Currently, there is no comparison of existing FWS guidelines.

Methods

This comparative review describes consistencies and differences in guideline definitions and diagnostic and therapeutic recommendations. A literature search was performed to include secondary care FWS guidelines of high-income countries, composed by national or regional pediatric or emergency care associations, available in English or Dutch.

Results

Ten guidelines of five high-income countries were included, with variating age ranges of children with FWS. In children younger than one month with FWS, the majority of the guidelines recommended laboratory testing, blood and urine culturing and antibiotic treatment irrespective of the clinical condition of the patient. Recommendations for blood culture and antibiotic treatment varied for children aged one to three months. In children aged above three months, urine culture recommendations were inconsistent while all guidelines consistently recommended cerebral spinal fluid testing and antibiotic treatment exclusively for children with a high risk of serious infection.

Conclusions

We found these guidelines broadly consistent, especially for children with FWS younger than one month. Guideline variation was seen most in the targeted age ranges and in recommendations for children aged one to three months and above three months of age. The findings of the current study can assist in harmonizing guideline development and future research for the management of children with FWS.

INTRODUCTION

Fever is one of the most common pediatric presentations at the emergency department.[1-3] While most children recover spontaneously without treatment from a self-limiting infection, serious infections can be harmful with long-term sequelae and mortality as potential consequences. The incidence of serious bacterial infections in children up to three months is estimated at 8%, in neonates even higher at 9% to 13%.[4-6] Fever without a source (FWS), defined as acute fever since less than 7 days without a clear focus of infection after a complete examination, is challenging for physicians as children often present with nonspecific symptoms and the initial clinical presentation can vary widely.[7] This can result in a delay in admission and treatment for serious infections. Multiple laboratory tests in search for a cause of FWS and empirical antibiotics are therefore applied frequently.[8]

To overcome this diagnostic challenge, several countries have developed guidelines to guide physicians in the management and evaluation of FWS.[8] They aim to detect those children at risk for serious infections requiring immediate treatment while avoiding overuse of unnecessary investigations and therapies. Despite the availability of guidelines for both children with a presumed low or high risk of serious infection based on the clinical condition, the variability in definition and management of FWS is significant.[9-13] For example, hospital admission and cerebral spinal fluid (CSF) analysis rates vary across hospitals from 40% to 90% of children presenting with FWS.[14] This variation in practice could be explained by several factors, including inconsistency in diagnostic and treatment recommendations between FWS guidelines or low adherence to these guidelines.[15]

The aim of this study was therefore to compare the definitions and the diagnostic and therapeutic recommendations published in national and regional FWS guidelines of high-income countries. Identifying differences between national guidelines with similar health care settings could improve harmonization of practice recommendations and inform future guideline development. With this approach, we aim to increase international consensus in high-income countries in definition and management of FWS.

METHODS

Study design

A literature search was performed to identify national and regional FWS guidelines in children in high-income countries. Since there is no universal consensus on health care system quality classification, we defined several criteria to include high-income countries with comparable health care systems: (1) classified as high-income economy level by the 2020 World Bank classifications; (2) rated in the top 50 countries with highest life expectancy; and (3) an antibiotic drug resistance index of 50 or less.[16-18]

The search was conducted on April 21th 2021, combining the search terms “guideline”, “child” and “fever” in the databases of PubMed, Web of Science and EBSCOhost, including variations of these terms (supplementary table 1). Only the most updated versions of guidelines were included. References of included articles were screened for eligibility using the forward and backward snowball method. Articles were included if a national or regional guideline described recommendations or considerations for children with FWS aged 0-18 years, including both children with a presumed low or high risk of serious infection based on the clinical condition. The recommendations had to describe FWS management, aiming to timely diagnose and treat serious infections. Guidelines had to be composed by national or regional pediatric or emergency care associations, health institutes, health networks or statewide health services and based on peer reviewed evidence or group consensus. The exclusion criteria were specified as follows: (1) local guidelines of hospitals; (2) primary care guidelines; (3) guidelines describing fever with a focus, fever of unknown origin (fever > seven days), early onset neonatal sepsis (within 72 hours after birth), a hospital-acquired fever or post-operative fever; (4) inclusion of both adults and children; (5) guideline not available in English or Dutch.

Outcome parameters

Primary outcome was to describe consistencies and differences in (1) guideline definitions; (2) diagnostic recommendations; and (3) therapeutic recommendations of included guidelines. Guideline definitions were described for age of population, fever, FWS, potential serious infections and objectives. Diagnostic recommendations were compared for laboratory testing of white blood cell count (WBC), C-reactive protein (CRP), performing blood culture, urine culture, CSF analysis, and polymerase chain reaction assay (PCR) for viral pathogens such as influenza virus, respiratory syncytial virus, enterovirus and parechovirus. Therapeutic recommendations were compared for empirical intravenous antibiotic treatment and empirical intravenous acyclovir treatment. Empirical antibiotic treatment is defined as antibiotics that are administered prior to the identification of the causing pathogen.

We divided diagnostic and therapeutic recommendations into those advised to perform or those advised to consider. To further specify the target population, categories were established a priori to distinguish recommendations based on age irrespective of the clinical condition or based on age combined with clinical criteria. Categories were defined as follows: recommendations (1) advised for all children irrespective of the clinical condition; (2) advised for children with a high risk of infection; (3) advised for children with an intermediate risk of infection; or (4) advised for children with a low risk of infection. Clinical criteria for low, intermediate or high risk of serious infection were described per guideline.

RESULTS

A total of ten guidelines were included, four from Australia, three from the United States of America and one from the Netherlands, the United Kingdom and Canada, respectively (table 1). The flowchart of the search is presented in supplementary figure 1. The publication year of these guidelines ranged from 1993 to 2021. Five of ten guidelines reported an established method to grade the quality of evidence supporting their recommendations.[19,20,22-24] Six guidelines were composed by national associations or health institutes.[19-23,28]

Country	Guideline	Year of publication
The Netherlands	Fever in children [19]	2013
UK	Fever in under 5s: assessment and initial management [20]	2019
USA	Management of fever without source in infants and children [21]	2000
	Practice guideline for the management of infants and children 0-36 months of age with fever without source [22]	1993
	Evaluation and management of well-appearing febrile infants 8 to 60 days old [23]	2021
Australia	Children and infants with fever [24]	2020
	Fever in children aged 1-2 months [25]	2020
	Febrile illness: emergency management in children [26]	2019
	Fever without source [27]	2020
Canada	Fever in young infants [28]	2019

Table 1 Guideline characteristics
UK: United Kingdom; USA: United States of America; NVK: Nederlandse Vereniging voor Kindergeneeskunde; NICE: National Institute for Health and Care Excellence; AAP: American Academy of Pediatrics; ACEP: American College of Emergency Physicians; NSW: New South Wales; SA: South Australian; CHQ: Children's Health Queensland Hospital and Health Service; CAHS: Child and Adolescent Health Service; TREKK: Translating Emergency Knowledge for Kids. AGREE II: Appraisal of Guidelines for Research & Evaluation Instrument; GRADE: Grading of Recommendations Assessment, Development and Evaluation; AHRQ: Agency for Healthcare Research and Quality's; NHMRC: national health and Medical Research Council.

Organisation	Approach to rate quality of evidence
Dutch Association of Pediatrics (NVK)	AGREE II
National Institute for Health and Care Excellence (NICE)	GRADE
American College of Emergency Physicians (ACEP)	None
American Academy of Pediatrics (AAP)	Modified Delphi
American Academy of Pediatrics (AAP)	AHRQ
New South Wales (NSW) government	NHMRC designation of levels of evidence
South Australian (SA) Pediatric Clinical Practice	None
Children's Health Queensland Hospital and Health service (CHQ)	None
Government of Western Australia – Child and adolescent Health service (CAHS)	None
Translating Emergency Knowledge for Kids (TREKK)	None

GUIDELINE DEFINITIONS

An overview of the guideline definitions is shown in table 2. The age range of the population as reported by the guidelines varied widely. The target age population of two guidelines was zero to two months, compared to zero months to sixteen years according to the Dutch guideline.[19,23,28] Fever was defined as a temperature of ≥ 38.0° by seven of ten guidelines.[20-23,25,26,28] There was no definition of FWS described in seven of ten guidelines.[19,20,23-26,28] The guideline was applicable to both children with a low risk and a high risk of serious infection in nine of ten guidelines.[19-22,24-28] The guideline of the American Academy of Pediatrics (AAP) was the only guideline applicable exclusively to children with a low risk of serious infection.[23] Recognizing serious infection was an objective in seven of ten guidelines.[19,20,22,24-26,28]

Table 2 (next page) Guideline definitions

UK: United Kingdom; USA: United States of America; NVK: Nederlandse vereniging voor kindergeneeskunde; NICE: National Institute for Health and Care Excellence; AAP: American Academy of Pediatrics; ACEP: American College of Emergency Physicians; NSW: New South Wales; SA: South Australian; CHQ: Children's Health Queensland Hospital and Health Service; CAHS: Child and Adolescent Health Service; TREKK: Translating Emergency Knowledge for Kids.

* Definition in broad terms, may differ from the precise definition in the guideline.

Country	The Netherlands	UK	USA		
Guideline	**NVK**	**NICE**	**ACEP**	**AAP**	**AAP**
	[19]	**[20]**	**[21]**	**[22]**	**[23]**
Age of population					
0 - 2 months					X
1 - 2 months					
0 - 3 years			X	X	
0 - 5 years		X			
0 - > 3 months					
0 - 16 years	X				
Definition fever					
T ≥ 38.0 C		X	X	X	X
T > 38.0 C	X				
Definition fever without source					
None available	X	X			X
No source of infection is apparent after a thorough examination in a nontoxic infant or child without significant underlying illness			X		
An acute febrile illness in which the etiology of the fever is not apparent after a careful history and physical examination*				X	
Target population FWS					
Applicable for low risk group only					X
Applicable for high risk group only					
Applicable for high and low risk group	X	X	X	X	
Definition serious infection					
None available					X
Meningitis	X	X	X		
Sepsis	X			X	
Bacteremia			X		
Urinary tract infection	X	X	X	X	
Pneumonia	X	X	X	X	
Enteritis				X	
Septic arthritis	X	X		X	
Osteomyelitis	X	X		X	
Encephalitis	X	X			
Kawasaki	X	X			
Objectives					
Recognize serious infection	X	X		X	
Minimizing diagnostics	X				
Evidence management FWS			X		X
Improve clinical assessment FWS		X			X
Decrease variation in care					X

Australia NSW [24]	SA [25]	CHQ [26]	CAHS [27]	Canada TREKK [28]
				X
	X			
X				
		X	X	
	X	X		X
X			X	
X	X	X		X
			X	
X	X	X	X	X
			X	X
X	X	X		
X			X	
	X	X		
X	X	X		
	X	X		
	X			
	X	X		
	X	X		
X		X		
X	X	X		X
X		X		X
				X

To distinguish recommendations for children with a low, intermediate or high risk of serious infection, the guidelines used multiple clinical criteria (supplementary table 2). We found a wide variation in clinical criteria. Most guidelines defined a high risk of serious infection in case of a pale or mottled skin, lethargy or drowsiness, grunting or tachypnea.[19,20,24-27] Criteria mentioned as classifying for a low risk are less consistent, but mostly included birth after 37 weeks of gestation and a non-toxic clinical condition. In total, 20 clinical criteria were mentioned defining the low risk group, 25 defining the intermediate risk group and 36 defining the high risk group.

Country	The Netherlands	UK	USA		
Guideline	**NVK**	**NICE**	**ACEP**	**AAP**	**AAP**
	[19]	**[20]**	**[21]**	**[22]**	**[23]**
CRP					
< 1 month	< 13 days		*	*	8-22 days
	> 13 days				22-28 days
1-3 months			*	*	
> 3 months			*	*	
WBC					
< 1 month	< 13 days				8-22 days °
	> 13 days				22-28 days °
1-3 months					°
>3 months	X				
Blood culture					
< 1 month	< 13 days				8-22 days
	> 13 days				22-28 days
1-3 months					
> 3 months					
Urine culture					
< 1 month	< 13 days				8-22 days
	> 13 days				22-28 days
1-3 months					
> 3 months					
CSF analysis					
< 1 month	< 13 days				8-22 days
	> 13 days				22-28 days
1-3 months					
> 3 months					
PCR viral					
< 1 month	< 13 days	*	*	*	*
	> 13 days				
1-3 months		*	*	*	*
> 3 months		*	*	*	

Diagnostic guideline recommendations

Diagnostic recommendations of each guideline are shown in table 3. There are a number of consistencies between the guidelines. For all children irrespective of the clinical condition younger than one month of age and one to three months old, laboratory testing of WBC or CRP was recommended by most guidelines.[19,20,25-28] Furthermore, seven of nine guidelines recommended a blood and urine culture in all children younger than one month.[20-22,24,26-28] In children older than three months, guidelines recommended performing a blood culture[20-22,24,26,27] and considering CSF analysis exclusively in case of a high risk of serious infection.[19,20,24,27]

Table 3 (previous page) Diagnostic recommendations and considerations
Abbreviations: UK: United Kingdom; USA: United States of America; NVK: Nederlandse vereniging voor kindergeneeskunde; NICE: National Institute for Health and Care Excellence; AAP: American Academy of Pediatrics; ACEP: American College of Emergency Physicians; NSW: New South Wales; SA: South Australian; CHQ: Children's Health Queensland Hospital and Health Service; CAHS: Child and Adolescent Health Service; TREKK: Translating Emergency Knowledge for Kids.; PCR: polymerase chain reaction; WBC: white blood cell; CRP: C-reactive protein; °: absolute neutrophil count.

Legend table 3 (previous page) and table 4

- recommended to perform for children with a low risk of serious infection
- recommended to consider for children with a low risk of serious infection
- recommended to perform for children with an intermediate risk of serious infection
- recommended to consider for children with an intermediate risk of serious infection
- recommended to perform for children with a high risk of serious infection
- recommended to consider for children with a high risk of serious infection
- recommended to perform for all children irrespective of clinical condition
- recommended to consider for all children irrespective of clinical condition

* not mentioned
X specifically discouraged
Empty box = not applicable

Besides consistencies, we also found a number of differences in diagnostic recommendations. In children between one to three months old, six guidelines[20,21,24,26-28] recommended to perform blood culture irrespective of the clinical condition compared to three guidelines[19,22,25] who recommended to perform blood culture exclusively in case of a high risk of serious infection. There was also disagreement in urine cultures in children older than three months with some guidelines only recommending a urine culture for children with a high risk of serious infection[20-22,24,26],

Country	The Netherlands	UK	USA		
Guideline	NVK [19]	NICE [20]	ACEP [21]	AAP [22]	AAP [23]
AB IV					
< 1 month	< 13 days				8-22 days
	> 13 days				22-28 days
1-3 months					
> 3 months					
Acyclovir IV					
< 1 month	< 13 days		*	*	8-22 days
	> 13 days				22-28 days
1-3 months			*	*	
>3 months			*	*	

Table 4 therapeutic recommendations and considerations
Abbreviations: UK: United Kingdom; USA: United States of America; NVK: Nederlandse vereniging voor kindergeneeskunde; NIC
Emergency Physicians; NSW: New South Wales; SA: South Australian; CHQ: Children's Health Queensland Hospital and Health S
intravenous.

while other guidelines recommended a urine culture irrespective of the clinical condition.[19,27] In children between one to three months old, recommendations for CSF analysis varied from considering it in all children or considering in children with high risk of serious infection, to performing in all children with high risk of serious infection. Also, three of ten guidelines recommended to perform PCR for viral pathogens[19,25,28], while seven guidelines do not mention any recommendations.[20-24,26,27] Finally, instead of CRP measurement, three of ten guidelines described the diagnostic value of incorporating procalcitonin in future guidelines and advise further research.[19,20,28]

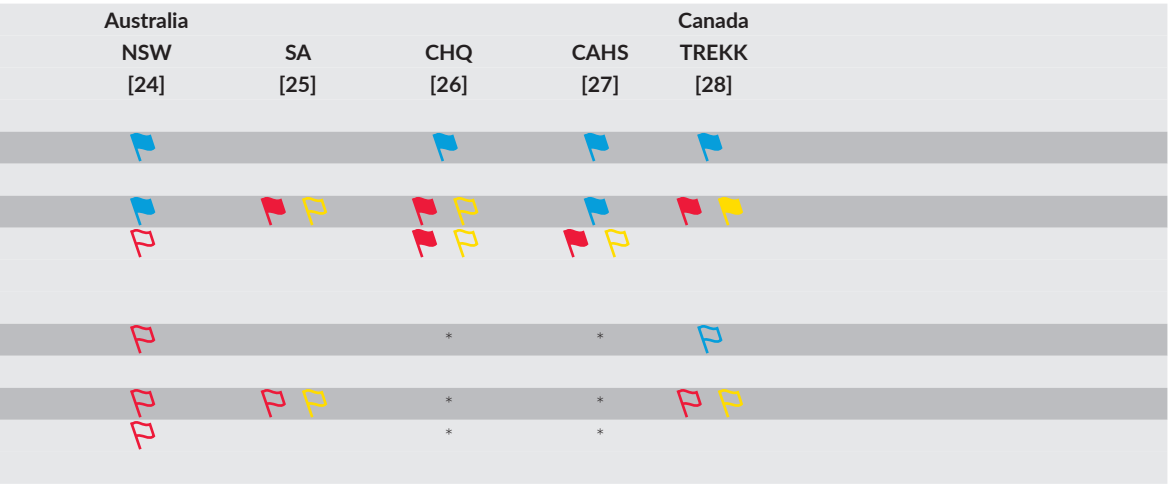
Therapeutic guideline recommendations

Therapeutic recommendations of each guideline are shown in table 4. Almost all guidelines recommended antibiotic treatment for children younger than one month old, irrespective of the clinical condition. [19-22, 24-28] There was agreement for antibiotic treatment in children older than three months: all guidelines recommended to treat or consider antibiotic treatment exclusively in case of high risk of serious infection. [19-28] Differences in antibiotic treatment was seen in children one to three months old. Seven guidelines recommended antibiotic treatment in children with high risk of serious infection, compared to two guidelines who recommended antibiotic treatment in children irrespective of the clinical condition. [19-22, 25, 26, 28] Four guidelines did not mention acyclovir treatment [21, 22, 26, 27], while six guidelines recommended to consider acyclovir treatment only in children with high risk of serious infection, irrespective of age. [19, 20, 23-25, 28]

DISCUSSION

In this study we compared the definitions and diagnostic and treatment recommendations of national and regional FWS guidelines of five high-income countries. We found these guidelines broadly consistent, especially for children younger than one month. The reported age range of children with FWS varied widely. Differences were seen most in recommendations for children aged one to three months and above three months of age in performing microbiologic cultures, CSF analysis and in antibiotic treatment. This knowledge may be of assistance to future guideline development.

We found consistency across the included FWS guidelines, particularly in children younger than one month with FWS with most guidelines advising CRP testing and antibiotic treatment irrespective of the clinical condition.



E: National Institute for Health and Care Excellence; AAP: American Academy of Pediatrics; ACEP: American College of Service; CAHS: Child and Adolescent Health Service; TREKK: Translating Emergency Knowledge for Kids.; AB: antibiotics; IV:

This agreement in managing young children is also reflected in clinical practice. Among 37 emergency departments in the United States most consistency was reported in laboratory testing in children younger than one month, compared to substantial variation in children aged one to two months and two to three months. A similar inverse association between age and practice variation in antibiotic treatment was reported by Aronson et al..[9] Our study found agreement among all guidelines in antibiotic treatment of children with high risk of serious infection younger than three months of age. Moreover, consistency was seen in a sepsis work-up for children older than three months of age meaning all guidelines recommended to perform or consider a blood culture, CSF analysis and antibiotic treatment exclusively in children with a high risk of infection. These findings implicate that most guidelines adopt a similar careful approach in neonates while advising a higher threshold to extensive diagnostic and therapeutic management in children aged above three months. This approach is understandable, considering the higher risk of bacterial infection in neonates compared to older children.[4-6]

The results of this study also show important differences between FWS guidelines, particularly for children older than one month. In children aged above three months there was particular disagreement in when to perform basic diagnostic testing, whereas in children aged one to three months guidelines were inconsistent in when to perform a sepsis work-up. This is in line with the previously mentioned variation in performed CSF analysis, with rates ranging between hospitals from 40% to 90% of children with FWS aged one to three months.[14,29] This guideline inconsistency and concurrent practice variation reflect the diagnostic dilemma of the age category in between the young neonate with an elevated risk of serious infection, and the older child with a lower risk and a decreasing trend in extensive diagnostic testing.[30] Guidelines may partially differ due to geographic differences in primary and secondary health care systems, antibiotic use and resistance patterns. Weighing risks and benefits of extensive testing and empirical antibiotic treatment may also be influenced by cultural opinions and preferences of physicians and parents.[31,32] However, we also reported differences between guidelines from the same country. Another reason for differences between guidelines is the lack of international consensus in definitions of FWS, potential serious infections and relevant age ranges. This lack has not been addressed in literature regarding FWS as much as for neonatal sepsis. Similarly for neonatal sepsis, lack of consensus in definitions of FWS hampers ongoing collaborative research and benchmarking for guideline development.[33] For instance, the targeted age range varied widely. Despite multiple studies reporting a drastic step-wise decrease of serious bacterial infection after the first week of life, most guidelines still classified all children younger than one month as high risk.[23] Third, the development and implementation of new diagnostic methods also contributes to differences: the use of PCR to detect viruses for example was only mentioned by a few guidelines. As (respiratory) viruses are a frequent cause of FWS, overuse of antibiotics is likely to decrease when viral testing is addressed in FWS guidelines and should therefore be included.[34-36]

Inconsistency between FWS guidelines has important consequences, contributing to increased practice variation. Aronson et al. evaluated the association between guideline inconsistency and practice variation among hospitals in the United States. The FWS recommendations from 21 separate hospital guidelines contained much variance, which correlated with the observed practice variation.[9] Moreover, adherence to FWS guidelines in the Netherlands was only 50% which indicates room for improving implementation of guideline recommendations or the recommendations themselves.[13] An Australian study showed a wide range of adherence across FWS recommendation categories and age groups.[37] They measured lower adherence in older children, where our findings stated most inconsistency between the Australian guidelines in children older than one month. Studies of barriers to guideline adherence reported several factors influencing physicians, including lack of agreement with recommendations, doubts about the scientific grounds or lack of outcome expectancy, complicated description of recommendations and inconsistency between similar guidelines.[15,38] Our findings corroborate several of these barriers in FWS guidelines, besides the inconsistency between guidelines. The majority did not report an established method of grading scientific evidence supporting their recommendations, which may increase doubts among physicians. While most guidelines were updated in the last two years, the AAP guideline for FWS applicable to children with both a low and high risk for serious infection was published in 1993.[22] Recently, the AAP published a new guide-

line applicable for the well-appearing child with FWS, yet an updated guideline applicable for children with a high risk is still lacking.[23] This is likely to contribute to the aforementioned variance between the 21 separate FWS hospital guidelines.[9] Therefore, our findings and these studies indicate several aspects that could improve guideline adherence such as decreasing inconsistency between guidelines–particularly within countries–using established grading methods and regularly updating guidelines.

The findings of the current study can assist in harmonizing guideline development and future research for the management of children with FWS. Despite many publications on risk assessment tools and practice guidelines, the appropriate management of children with FWS still remains a highly debated and studied topic. In guideline development, it is common to perform a search of existing guidelines regarding specific management and compare it with the latest evidence to compose a recommendation. Subsequently, the aim is to provide evidence-based practical guidelines, improving quality of care and reducing unwanted variation. It is not necessary or advisable to aim for complete harmonization between national guidelines, as practical considerations and local applicability are also taken into account. Differences between health care systems or resistance patterns in high-income countries can provide solid arguments for international differences between guidelines. It is, however, very likely that many recommendations are based on the same available evidence. To support interpretation and comparison of evidence for guideline development, it is recommended to establish international consensus on targeted age groups and definition for FWS and potential serious infections. Furthermore, identifying significant differences between guidelines provides insight in FWS recommendations lacking consensus or lacking valid scientific grounds and may reveal important opportunities for further analysis and increasing adherence.

We acknowledge that this study contains several limitations. Although our literature search enabled a comparison of guidelines from various countries, we may have missed potential eligible guidelines due to the exclusion of non-English or Dutch guidelines and possible lack of access to guidelines or guidelines which are not published publically. We did not use translation programs to include guidelines in more languages, since the interpretation of health care recommendations require a detailed understanding of the language and may be prone to mistakes. However, our detailed description of FWS definitions and recommendations can easily be compared to a physician's own local guidelines. Furthermore, our study does not include a guideline quality assessment. This was a deliberate decision since our aim was not to compare the quality of guidelines, but rather to provide insight in current existing recommendations and reveal important differences between guidelines.

In conclusion, national and regional FWS guidelines of high-income countries for management of children are broadly consistent. However, substantial differences were found in diagnostic and treatment recommendations for children aged one to three months and above three months. In the context of considerable variation in current practice and guideline adherence, our results imply a need for consistent, effective and practical recommendations for children with FWS aged older than one month. International consensus in age range, definition and management of FWS could improve future guideline development and research efforts. Further research should be undertaken to investigate what scientific or practical reasoning drives the differences between guidelines and evaluate if consensus between guidelines is needed.

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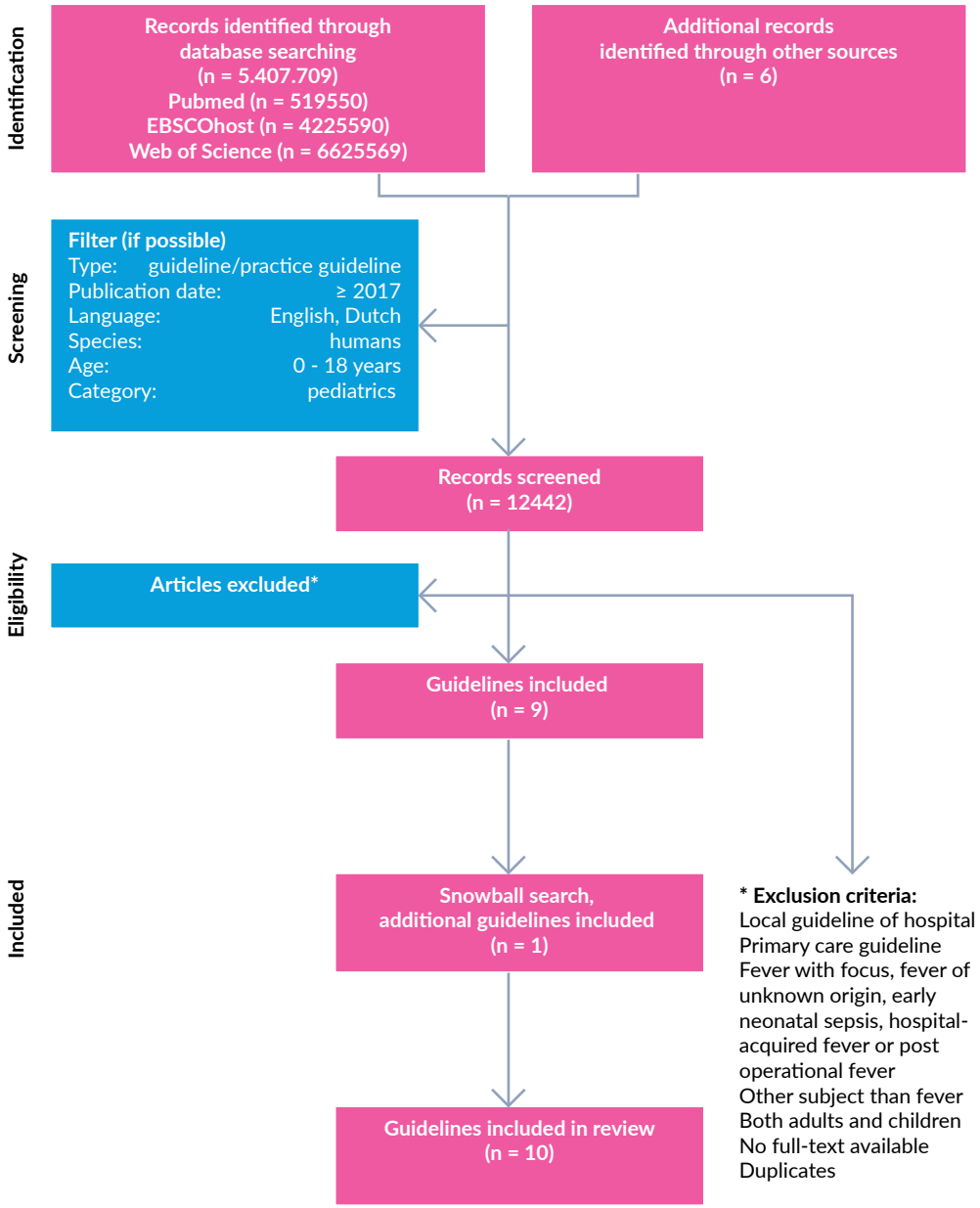
SUPPLEMENTARY MATERIAL



Supplementary Figure 1 Flowchart of the literature search

Concept	Search terms
Children	"infant" OR "newborn" OR "neonate" OR "neonates"
	OR "baby" OR "babies" OR "infant"
	OR "Children" OR "Child" OR "minors" OR "pediatric"
Fever	"Fever" OR ''febril" OR ''febrile" OR ''hyperterm"
Guideline	"Guideline" OR "Guidelines"

Supplementary Table 1 search terms for Pubmed and Google Scholar database search. The concepts were combined as follows: ''children'' AND ''fever'' AND ''guideline".

Country	The Netherlands	UK	USA
Guideline	NVK [19]	NICE [20]	ACEP [21]
Low risk			
Normal color of skin, lips and tongue	X	X	
Responds normally to social cues	X	X	
Content/smiles	X	X	
Stays awake of awakes quickly	X	X	
Strong normal cry of not crying	X	X	
Normal work of breathing			
Normal skin and eyes	X	X	
Moist mucous membranes	X	X	
Previous healthy			X
Born after 37 weeks of gestation	X	X	X
No prior hospitalization			
No prolonged newborn nursery care			
Uncomplicated newborn nursery care			X
Nontoxic clinical appearance	X	X	X
No intermediate or high risk signs	X	X	
No focal bacterial infection on examination (except otitis media)	X	X	X
No evidence of any infection clinically	X	X	
No unexplained jaundice	X	X	
No chronic illness	X	X	
No prior antibiotics	X	X	
Intermediate risk			
Pallor of skin, lips or tongue, or reported by parent or carer	X	X	
Not responding normally to social cues	X	X	
No smile	X	X	
Wakes only with prolonged stimulation	X	X	
Decreased activity	X	X	
Irritable			
Not strong cry			
Appearing ill to a healthcare professional	X		
Disease course different from previous diseases	X		
Nasal flaring	X	X	
Tachypnea	X	X	
Oxygen saturation ≤95%	X	X	
Auscultation: crepitations	X	X	
Tachycardia	X	X	
Dry mucous membranes	X	X	
Poor feeding in infant	X	X	
Reduced urine output	X	X	
Capillary refill time ≥ 3 s	X	X	
Rigors	X	X	
Fever >5 days	X	X	
Swollen joint	X	X	

		Australia				Canada	
AAP [22]	AAP [23]	NSW [24]	SA [25]	CHQ [26]	CAHS [27]	TREKK [28]	
			X		X		
			X		X		
			X		X		
			X		X		
			X		X		
			X		X		
			X		X		
			X		X		
X							
			X			X	
						X	
						X	
X							
					X		
X							
			X				
						X	
			X			X	
			X			X	
			X	X	X		
			X	X	X		
			X	X	X		
				X	X		
			X	X			
			X				
				X			
				X	X		
				X	X		
				X	X		
				X	X		
				X	X		
			X		X		
			X	X	X		
			X	X	X		
				X	X		
				X	X		
					X		
				X	X		

Country	The Netherlands		UK	USA
Guideline		NVK [19]	NICE [20]	ACEP [21]
New lump > 2 cm				
Non-use of a limb		X	X	
Unable to bear weight		X	X	
Age 3-6 months, temperature ≥ 39.0 C			X	
High risk				
Pale/mottled/ashen/blue skin, lips or tongue		X	X	
No response to social cues		X	X	
Altered mental state				
Lethargy				
Irritability				
Decreased activity				
Decreased alertness				
Appearing ill to a healthcare professional			X	
Does not wake or if roused does not stay wake		X	X	
Weak, high-pitched or continuous cry		X	X	
Grunting		X	X	
Hypoventilation or hyperventilation				
Tachypnea		X	X	
Moderate or severe chest indrawing		X	X	
Auscultation: diminished breath sounds				
Signs of poor perfusion				
Cool peripheries				
Bounding pulses or wide pulse pressure				
Reduced skin turgor		X	X	
Bilious vomiting				
Decreased fluid intake				
Decreased urine output				
Tachycardia				
Signs of shock				
Neck stiffness		X	X	
Bulging fontanelle		X	X	
Petechiae		X		
Non-blanching rash			X	
Coagulopathy				
Rigors				
Status epilepticus	X	X		
Focal seizures	X	X		
Focal neurological symptoms	X	X		
Age <1 month with FWS	X			
Age <3 months with FWS		X		
Evidence of organ dysfunction				

Supplementary Table 2 Overview clinical criteria for low, intermediate and high risk of serious disease per guideline

		Australia				Canada	
AAP [22]	AAP [23]	NSW [24]	SA [25]	CHQ [26]	CAHS [27]	TREKK [28]	
			X				
					X		
					X		
					X		
X		X	X		X		
			X		X		
				X			
X		X		X		X	
		X		X			
		X					
		X					
			X		X		
			X		X		
		X	X	X	X		
			X	X	X		
X							
		X	X	X	X		
		X		X	X		
X		X		X			
			X	X			
			X				
			X	X	X		
			X				
		X	X	X			
		X	X	X			
		X	X	X			
						X	
					X		
			X		X		
			X				
					X		
						X	
			X				
				X			
		X	X	X			
				X			
				X			
						X	

3

General discussion & appendices

Chapter X

General discussion

This thesis aims to describe challenges and explore opportunities for improving diagnosis of children with suspected infections. Specifically, challenges of recognition and sample collection have been evaluated from the perspective of congenital syphilis, neonatal herpes simplex virus (HSV) infection and pediatric severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. From the perspective of children presenting with fever without a source (FWS), we have evaluated challenges of the current Dutch national FWS guideline. We have summarized and discussed our findings in this chapter, and have provided opportunities and future perspectives for improving diagnosis in suspected pediatric infection.

OBJECTIVE A: RECOGNITION OF SEVERE PEDIATRIC INFECTIONS

Challenges of recognition

Clinical presentations of pediatric infections can be nonspecific, varying per case or mimicking other diseases. Although **chapters 2 and 3** on congenital syphilis and neonatal HSV infections are limited by their small sample size, they demonstrate that rare yet severe infections are prone to variation in practice. A certain amount of variation in practice of rare infections is inevitable, and can be explained by differences in clinical presentation and illness severity.[1] When delay in recognition leads to a preventable and harmful delay in diagnosis and treatment, for instance in congenital syphilis and in neonatal HSV infection, this requires further attention.[2, 3] In the case described in **chapter 2**, early recognition and antibiotic treatment may have prevented the manifestations of portal hypertension and respiratory deterioration that have led to long-term intensive care. In our neonatal HSV case series we detected delays in testing of two or more days, particularly in neonates presenting without fever, without skin lesions or with unrecognized skin lesions. Another case series of neonatal HSV infection with only non-vesicular skin lesions described a wide variation in morphology and frequent diagnosis delay as well.[4] Skin manifestations included, among others, ulcerations, papules, excoriations, macules, atrophy, contractures, and bruising. Most likely, the diverse morphology of skin manifestations of both congenital syphilis and neonatal HSV has led to diagnosis delay.

Another important contributing factor to late recognition of rare infections is unfamiliarity of physicians with specific disease manifestations. While the variation in skin manifestations is often described in syphilis – hence the nickname 'the great imitator' – , vesicular lesions are generally the only mentioned characteristic of HSV infections.[5] A survey among senior pediatric residents found that HSV infection was often not considered in the differential diagnosis of ill-appearing neonates when vesicular lesions were absent.[6] Skin lesions can present with different morphology or be absent, as was the case in half of our HSV infections. Awareness regarding the diverse morphology of HSV related skin manifestations is crucial for optimal diagnosis and treatment. Similarly, awareness of the value of other clinical or laboratory predictors such as fever, which was absent in the majority of patients, is mandatory as well. Regarding familiarity with syphilis among physicians with varying levels of experience, 96% reported feeling inexperienced with its diagnosis and treatment.[7] The Netherlands has an antenatal screening coverage of > 99%, with a low prevalence of maternal syphilis (0.06 – 0.08%) and only a few cases of congenital syphilis yearly.[8] Subsequently, the very low incidence of congenital syphilis has decreased familiarity of physicians with this disease. **Chapter 2** is an illustrative example of this, with the misinterpretation of the possibility of vertical syphilis transmission after antenatal screening. In this case, the unawareness of syphilis transmission after antenatal screening led to exclusion of congenital syphilis from the differential diagnosis. Ours was not an exception: several high-income countries reported missed severe congenital syphilis cases in children of mothers testing negative in antenatal syphilis screening.[9-13] Although universal third trimester syphilis screening would have detected our case, this approach is likely not cost-effective in the Netherlands. As our congenital syphilis rate is already very low and eliminating all risk is improbable while syphilis still circulates in the population, cost-effectiveness of new approaches is of great importance. An analysis from the United Kingdom, with a maternal syphilis prevalence of 0.04% resembling our prevalence rates, concluded that universal syphilis screening in the third trimester is not cost-effective in such a low prevalence setting.[14] For other high-income countries the reported increasing congenital syphilis rates imply the need to reconsider their screening approach.[15] Despite the high antenatal screening

coverage in the Netherlands congenital syphilis has not yet been eliminated and therefore it remains relevant to consider this disease in challenging infectious patients and recognize its spectrum of manifestations.
The development of prediction tools for recognition of severe or rare pediatric infections still faces many challenges. Development of prediction tools for rare infections is hampered by the low incidence, the large spectrum of manifestations and diverse disease morphology. Furthermore, although many prediction tools for children with FWS have been proposed, there is no consensus on the relevant elements of these tools.[16, 17] As described in **chapter 9**, there is heterogeneity between FWS guidelines in the patient population and in clinical and laboratory predictors. Similarly, a European collaboration has stated wide variability in targeted patients, definitions of severe infections and number and type of predictors in their systematic review of studies on FWS prediction tools.[17] In anticipation of broadly accepted consensus definitions of the targeted population, the relevant severe infections and on the set of predictors, this heterogeneity will remain a barrier to implementation of an FWS prediction tool.[17]

Future perspectives

Improving recognition unquestionably requires the collaboration of research groups and hospitals to generate sufficient generalizable data and to increase awareness. With regard to the previously described challenges of recognition, we propose three main objectives for collaborative research networks. First, to evaluate incidence and predictors of (rare) severe infections in several populations. Second, to increase awareness for the pitfalls of diagnosing pediatric infections, particularly for rare infections. And third, to achieve consensus between research networks on definitions for children with FWS regarding the targeted population, infections and predictors for severe infection.

Development of prediction tools to improve recognition of very rare and diversely presenting infections is challenging. With regard to syphilis, prediction tools are mostly focused on risk factors for maternal infection or vertical transmission instead of recognizing a child with congenital syphilis.[18, 19] Maintaining effective screening strategies will likely outweigh the benefits of a clinical tool for recognizing congenital syphilis. Collaborative reporting on the spectrum of manifestations and predictors can, however, be of supportive value to identify the diagnostic clues for these exceptional cases. For HSV infection no cost-effective screening approach has been identified yet, and recognition of neonatal infection therefore remains very relevant.[20] The Dutch National Institute for Public Health and the Environment periodically monitored national incidence of neonatal HSV infection since 1981.[21] This national survey should be expanded with data collection regarding clinical predictors and current diagnostic practice and should also include congenital syphilis surveillance. Similar efforts are currently being undertaken in the United Kingdom.[22] After identification of incidence and patterns of predictors, developing a validated neonatal HSV infection prediction tool would be helpful. For example, in the United States a 23-center case-control study derived a risk prediction tool for invasive neonatal HSV infection accurately identifying infants at low risk.[23] The tool contained demographic, clinical, and laboratory predictors. When applying this tool to our population of **chapter 3** all invasive HSV infections would have been identified, suggesting potential to implement and evaluate this tool in the Netherlands. It should be determined to what extent a certain tool would minimize testing in low-risk patients when compared to the current FWS guideline and when weighed against the costs of missing a diagnosis.[24] Prospective validation will, however, certainly be a challenge considering the low incidence. Finally, although prediction tools may assist recognition, identification of all neonatal HSV cases is unlikely considering the varying presentations and, thus, also requires increased awareness among physicians.

Achieving national or regional collaboration networks for the rare pediatric infections mentioned in this thesis will additionally enhance awareness of these infections and their pitfalls in recognition. These research efforts can be combined with educational programs to counter the unawareness and unfamiliarity with disease that comes with the low incidence rates of congenital syphilis and neonatal HSV infection. Following our findings on the pitfalls of recognizing congenital syphilis, awareness should specifically be raised for potential late acquisition of syphilis after antenatal screening. In any challenging infectious disease case of an infant presenting with a skin

rash, congenital syphilis should be considered despite a negative antenatal screening in the mother. Moreover, the diverse clinical presentation in neonatal HSV infection should be emphasized: neonates can present without fever, without skin lesions or with diverse morphology of skin lesions.

For FWS, investigating disease incidence and patterns in clinical and laboratory predictors in a collaborative network aids the development of prediction tools. A fruitful example is the PERFORM network of European emergency departments. Since 2016 this research network has been collaborating to improve diagnosis of febrile children, developing a prediction tool for identifying severe infection.[25] Accuracy and efficacy of this prediction tool should be compared to the current Dutch FWS guideline or the National Institutes for Health and Care Excellence (NICE) guideline in a randomized controlled trial design. Specifically, a new tool needs to lead to a decrease of unnecessary testing and treatment without increasing missed severe infections compared to the current approach. If implemented in the new guideline, evaluation in practice is needed to evaluate improvement of diagnostic and therapeutic resource use outside a research setting. The authors mentioned that while their tool shows potential to optimize antibiotic use in FWS, the performance is poor in children with intermediate risk and thus research efforts for prediction tools should target this group.[25] Following consensus on definitions of the targeted population, severe infections and relevant predictors, these definitions should be implemented in the new FWS guidelines and broadly communicated through national pediatric communication platforms.

Main points

- Collaborative national reporting on the spectrum of manifestations and predictors of rare infections can be of supportive value to improve recognition and increase physician's familiarity with rare disease.
- Consensus between research networks is needed on definitions of the targeted population, of the relevant infections and relevant predictors in children with FWS.

OBJECTIVE B: SAMPLE COLLECTION IN SUSPECTED PEDIATRIC INFECTION

Challenges of sample collection

As shown in **chapter 3** for neonatal HSV infection and in **chapters 7 and 8** for FWS, there is substantial practice variation in sample collection for diagnostic testing. Non-adherence to the FWS guideline, resulted in less sample collection yet, importantly, with no differences in clinical outcomes. The constantly changing epidemiology of pathogens demands frequent reevaluation of the diagnostic workup of pediatric infection. An illustrative example can be found in the Rochester criteria, developed in 1985 to detect children at low risk for bacterial infection and still implemented in current guidelines.[26] The value of a white blood cell count between 5 and 15 x 10^9/L, a Rochester criteria, is being questioned since vaccination programs have decreased *Haemophilus influenzae* and pneumococcal infection rates.[27,28] In a 26-center cohort of 4313 febrile children aged 0 to 60 days, the positive and negative likelihood ratios of these thresholds to identify bacteremia or bacterial meningitis were very low.[29] As we identified in **chapter 7**, Dutch physicians indeed most often deviated from the guidelines on this criteria by withholding a full sepsis work-up when an abnormal white blood cell count was the only indicator. These findings suggest that physicians are continuously adjusting their daily practice and are an additional argument to update the current Dutch FWS guideline with regard to the value of white blood cell counts. Fortunately, the SARS-CoV-2 pandemic did not cause a substantial increase in hospital admissions among children in 2020 and 2021, yet other future emerging (viral) infections may again require reevaluation of the current guidelines. Moreover, the development of new rapid diagnostic tests and infection biomarkers continue to change the optimal diagnostic workup of pediatric infection.

In the broader view of evaluating non-invasive sampling in pediatric infection, this thesis focuses on a method for immunosurveillance using saliva as an alternative to serum. Both locally produced and systemically derived antibodies can be detected in the mucosa.[30] Although the mucosa of the upper respiratory tract is the primary entry site of respiratory viruses and local immune responses are essential to combat infection, mucosal immunity is still

poorly understood.[31] **Chapters 5 and 6** describe how SARS-CoV-2 specific antibodies were detected in saliva of children. In the 2021 study antibody prevalence was significantly higher in serum compared to saliva. This lower sensitivity of saliva-based assays to detect PCR-positive previous infection compared to serum-based assays may be an obstacle in implementing saliva-based assays. Nonetheless, as the urgency of the pandemic is decreasing due to vaccination programs and previous infections, the relevance of low-impact methods such as saliva-based assays for immunosurveillance purposes will increase as low-impact methods enhance the motivation to participate in these activities.

Chapters 5 and 6 emphasize the heterogeneity in humoral immunity with regard to exposure, isotypes, antigens, compartments (systemic and mucosal) and sex. After SARS-CoV-2 vaccination, correspondence between serum and saliva was higher compared to previous infection indicating a potential use for monitoring vaccine response. Furthermore, some children were antibody positive in saliva while seronegative and vice versa. Considering the small numbers, this could represent either false positives, or it could indeed be heterogeneity in immune responses, indicating that these compartments can operate independently. Agreeing with the latter hypothesis, most of our seronegative children with saliva antibodies reported clinical clues for SARS-CoV-2 exposure and other COVID-19 cohorts have similarly endorsed the possibility of mucosal antibodies in seronegative individuals.[32, 33] An Israeli nation-wide study measured antibody prevalence with various assays utilizing different methods, antigens and isotypes.[34] Despite repeated testing, 5% of symptomatic PCR-proven patients did not show seroconversion. This compartmentalization of immune responses may be even more relevant in children as they showed significantly lower seroconversion after infection compared to adults (38% compared to 62-80%, respectively) despite a similar clinical and virological profile.[35] It is evident that systemic antibodies do not provide the full picture of SARS-CoV-2 induced immunity. Indeed for example Steiner et al. identified SARS-CoV-2-reactive T cell responses in seronegative mild COVID-19 patients.[36] Seropositive and seronegative patients showed similar T-cell responses, with significantly higher reactive CD4+ T-cells in both groups compared to healthy controls. Therefore, although mucosal assays show lower sensitivity than serum to identify previous SARS-CoV-2 exposure, saliva-based assays have additional value to serum antibodies in identifying seronegative convalescent patients.

Chapter 6 shows higher odds ratios for saliva antibody positivity in females compared to males. In general, many sex-related differences in immunity are described. With regard to adaptive immunity, females show stronger antibody responses, higher basal Ig titers and B-cells than males and girls show higher vaccine responses compared to boys after for instance diphtheria, pertussis or rabies vaccinations.[37] As extensively reviewed by Klein and Flanagan, many studies provide evidence of genetic and hormonal mediators explaining sex-related differences in immunity.[37] In the context of SARS-CoV-2 infection, the decline of spike-specific and neutralizing antibodies after mild COVID-19 was faster in males compared to females, independent of age and body mass index.[38] A large cohort of hospitalized patients showed higher B- and CD4+ T-cells during SARS-CoV-2 infection and recovery and a sharper increase of RBD-specific IgG in females compared to males.[39] SARS-CoV-2 vaccination could lead to slightly higher antibody titers in females compered to males, yet efficacy did not seem to differ.[40, 41] These findings implicate a possible transient higher antibody response in females compared to males with males catching up in IgG titers at a later time point. Our study inclusion started just before the initiation of COVID-19 vaccination programs in children. Considering this short time frame, we hypothesized that our sex-related difference in mucosal antibodies may indeed be transient with equal IgG positivity between the sexes if measurements were repeated. Importantly, these studies were performed in adults and evidence on sex-related differences in children with SARS-CoV-2 infection remains scarce.

Future perspectives

Several opportunities can be identified to improve the use of sample collection for diagnostic testing. With regard to neonatal HSV infection, unwanted variation in sample collection could be minimized by providing practice recommendations. For instance, in **chapter 3** most HSV encephalitis cases presented without neurological symptoms. In line with proposed recommendations by Ahmad et al, both skin and cerebral spinal fluid samples there-

fore seem justified in case of clinical suspicion of HSV disease irrespective of neurological symptoms.[42] Attempting to improve diagnostic testing, several research groups are developing infection biomarker combination tests, novel viral and bacterial biomarkers as well as gene expression profiling.[28] mRNA biomarkers in febrile infants have shown promising preliminary results, with potential to distinguish viral from bacterial infection and from asymptomatic detection.[43] When these biomarkers have been externally validated for point-of-care application, it will likely change our diagnostic workup. The future FWS guideline needs to be kept up-to-date by modularly implementing these new biomarkers. Moreover, the next revision of the national FWS guideline, expected in 2023, should focus on our identified variations in sample collection to identify and anticipate bottlenecks of guideline implementation. Rapid viral testing is already available for influenza virus, SARS-CoV-2 and respiratory syncytial virus. While the current FWS guideline does not provide strict recommendations on viral diagnostic testing, **chapter 7 and 8** show that these are often performed in practice. In the preliminary data of our prospective study, rapid testing of endemic respiratory viruses provided a plausible source in 16% of children with FWS leading to less exposure to further sample testing and antibiotic treatment. Viral testing for enterovirus has also shown potential to shorten length of admission and antimicrobial treatment.[44, 45] Future FWS guidelines need to implement evidence-based guidance on the use of viral tests taking account of the current epidemiology of circulating viruses, bacterial coinfections and their potential predictors.

Immunosurveillance is an important tool to provide epidemiological data on circulating pathogens. As a potential source for non-invasive immunosurveillance methods as well as for future biomarkers of current or past infection, we need to improve our understanding of mucosal immunity. Although our prevalence studies are not directly generalizable to the epidemiology in the general pediatric population, we did explore mucosal in comparison to systemic immunity in children. Understanding mucosal immunity is not only relevant for SARS-CoV-2 but also for other respiratory viruses frequently causing pediatric infection. In the context of SARS-CoV-2: the heterogeneity between systemic and mucosal antibodies may indicate separate indications for serum- or saliva-based assays and require further investigation. With a comparable specificity to serum assays and a substantial reduction in invasiveness of sample collection, saliva-based assays can provide valuable information regarding the lower threshold of antibody-based immunity in various populations. Our findings also support the importance of sex-stratified analysis in future antibody studies and, in case of children, should consider information on sexual maturation. Importantly, as our studies were not primarily designed to evaluate sex-differences and we did not find sex-differences for all type of antibodies, this requires further exploration in multiple types of SARS-CoV-2 exposed pediatric cohorts.

Main points

- Future FWS guidelines need to implement evidence-based guidance on the use of viral testing taking into account the current epidemiology of circulating viruses, bacterial coinfections and their potential predictors.
- The heterogeneity between systemic and mucosal SARS-CoV-2 antibodies may indicate separate indications for serum- or saliva-based assays and require further investigation.

OBJECTIVE C: CLINICAL PRACTICE GUIDELINES FOR SUSPECTED PEDIATRIC INFECTION

Challenges of clinical practice guidelines

Our multicenter studies of the Dutch FWS guideline detected non-adherence in only 50% of children presenting with FWS. Although we could not perform national adherence detection, we were able to include multiple pediatric departments providing care for all types of FWS patients including multiple levels of care. While these are the first studies describing adherence to the Dutch guideline, adherence to the NICE guideline for FWS showed similar non-adherence across European emergency departments.[46, 47] Intentional or unintentional non-adherence can be affected by multiple factors. A systematic review categorized these factors in a framework as barriers affecting the physician's knowledge, attitudes or behavior (figure 1).[48] We deliberately did not survey reasons for

non-adherence as to not affect behavior during our study, thus we could only hypothesize to what extent lack of familiarity or agreement with recommendations was explaining non-adherence. Clues for guideline factors to non-adherence can be found in **chapters 7, 8 and 9**, describing both confusing recommendations and contradictory FWS guidelines. As the findings of **chapters 7 and 8** will be considered in the implementation plan of the current revision of the Dutch guideline, these factors may hopefully be reduced.

Our findings raise the question whether interventions need to be applied to increase adherence, or if these adherence rates are actually a symptom of decreased applicability of the current FWS guideline. In a review of intentional non-adherence, reasons for non-adherence were often justifiable, and did not impact quality of care. Even more so, well-substantiated lack of agreement with recommendations may lead to improved quality of care. Adherence to the United States FWS guideline was even lower than in Europe, but led to a lower hospitalization rate without increasing missed severe infections when physicians deviated from recommendations.[49] Multiple examples of guideline evaluation showed that strict adherence would have led to overuse of diagnostic or therapeutic resources when compared to the physician's clinical judgement.[50, 51] Considering that the FWS guideline was published in 2013 and is being revised, it is plausible that new relevant evidence has been affecting current practice. The new United Stated guideline published in 2022 suggests a less defensive approach of well-appearing febrile infants while the 2021 update of the NICE guideline remains to recommend their traffic light system and concurrent recommendations.[52, 53] As a high non-adherence rate to the FWS guideline did not lead to unfavorable outcomes, the FWS guideline can safely recommend less testing and treatment and thus decrease the burden of patients and the costs of emergency department resources.

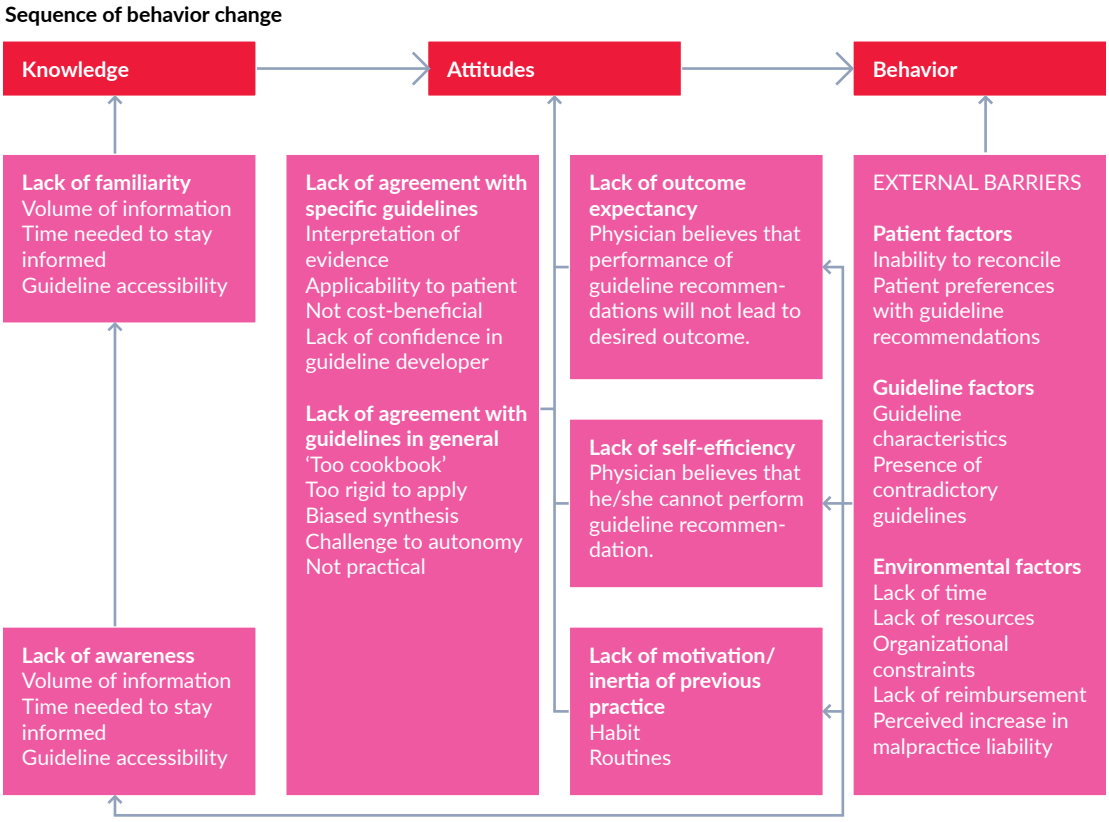


Figure 1 Barriers to physician adherence to guidelines in relation to behavior change.
Source: Cabana et al. JAMA 1999

Future perspectives

The diversity in clinical presentations, causing infections and diagnostic approaches in children presenting with FWS make these guidelines an eminent example of future guideline development opportunities. Certain amount of practice variation and guideline non-adherence is inevitable in the context of such diversity. The key is to find opportunities to achieve the most optimal balance between evidence-based support and the physician's judgement. With regard to future perspectives for guidelines in general, this will ultimately require finding a symbiosis of human and artificial intelligence (AI).

Perspectives for the FWS guideline

The current revision provides an opportunity to reduce non-adherence to the FWS guideline. Inclusion of our findings on non-adherence can aid anticipation of potential bottlenecks to guideline implementation or physician's lack of agreement in advance. The description of new recommendations should be clear and consistent and tested among guideline users on comprehensibility. Moreover, we propose a more substantial role for the physician's judgement. The Dutch FWS guideline categorizes physician's judgement only as an intermediately strong predictor of severe infection. In a multiple regression analyses, the physician's judgement was one of the strongest predictors of bacteremia, both in models with and without laboratory predictors.[49] Physician's judgement showed particularly higher specificity compared to the NICE guideline and thus decreased unnecessary testing.[54] These findings imply a reevaluation of the value of the physician's judgement. Modular updates should be performed in case of

new relevant clinical data, infection biomarkers or certified risk prediction tools for rare or severe infections. For example, a new national monitoring including clinical data is currently underway regarding the burden and approach of neonatal HSV infections. Lastly, feedback from the field should be automated using AI applications in order to improve guideline use while also accommodating the evaluation step of the guideline development cycle with minimal administrative efforts. As data on non-adherence could provide crucial information for revision, an automated feedback survey should be promoted and easily accessible for guideline users.

Perspectives for the role of guidelines

Considering future perspectives of clinical practice guidelines, AI prediction tools will likely play a pivotal role in improved recognition of severe and rare infections. If we find consensus on the population, severe infections and the predictors we should be able to develop a continuously deep-learning risk prediction algorithm integrating medical knowledge and data-driven modeling. This tool should provide a risk for severe or rare infection based on signs and symptoms collected through history taking and physical examination. These risk prediction tools should be built within the electronic medical record and thus easily accessible and fueled by automated data entry.
Although the first AI studies are already reporting similar or improved clinical performance compared to physicians, these tools have not been validated outside the research setting regarding accuracy and cost effectiveness.[55, 56] As AI tools are essentially built to classify in groups rather than to assess the risk for a single patient, there is an important difference between a predictive value for a study cohort and for the individual.[57] Moreover, as the optimal workup for an individual also requires weighing many subjective aspects and the physician's judgement is an important predictor for severe infection, we do not expect an AI prediction tool that can fully replace the physician and is applicable to every patient. On the other hand, the physician's judgement can be clouded by previous experiences with rare disease, overestimating its probability, or by unfamiliarity with rare disease presentation or optimal sample collection. We therefore need to develop guidelines informing physicians on the use of objective AI supported risk assessment in combination with the physician's subjective weighing of the individual context of the patient. In this way, subjective yet incorrect physicians' beliefs on risk will be diminished without eliminating their judgement. Thus, while AI tools will probably not fully replace the physician's own clinical judgement, it can provide less biased and extensively informed risk prediction leaving more time for the physician for communicating with the patient and finding an individualized approach.

Finally, we believe changing the framing of recommendations and improving transparency on their base of evidence will increase the physician's agreement with clinical practice guidelines and subsequent adherence. A distinction should be made between guideline components with and without a strong base of evidence. The components with a strong base of high-graded quality evidence consistently supporting a diagnostic or treatment recommendation can be implemented similar to current guidelines. For instance the use of an antimicrobial treatment regime based on several well-designed randomized controlled trials. The components that are less clear cut should move away from recommendations with 'low-grade quality evidence' or 'expert opinion' to simply providing the relevant underlying evidence and preferably a risk prediction tool. This calculated risk or underlying evidence can then be weighed in the subjective evaluation of the individual's context, meaning health care setting, patient preferences and abilities, the physician's judgement and change over time. Adherence should then only be measured regarding the strong evidence-based recommendations while the less clear components merely provide more background information or a risk calculation to support individual decision making.

Children presenting with FWS will continue to puzzle physicians in their recognition, sample collection and clinical practice guidelines. Future AI prediction tools have the potential to augment the physician's clinical reasoning instead of replacing it, and its use should be guided by clearly and transparently described clinical practice guidelines. Collaborative research networks are essential to continue finding pieces of the puzzle for improvement of the diagnosis in children with FWS.

Main points

- As the high non-adherence rate to the FWS guideline did not lead to unfavorable outcomes, a revised guideline can safely recommend less testing and treatment to decrease the burden on patients and the costs of emergency department resources.
- Continuously updating relevant clinical data for risk prediction algorithms, as well as acquiring feedback from the field should be automated using AI applications in order to evaluate and improve guideline use with minimal administrative efforts.
- Changing the framing of recommendations and improving transparency on their base of evidence will increase the physician's agreement with clinical practice guidelines and subsequent adherence.

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Chapter XI

Summaries

English summary

The Puzzle of Pediatric Infections

Challenges and opportunities for improving diagnosis of children with suspected infection

The puzzle of pediatric infection is to distinguish severe infections with potential life-threatening consequences from the many self-limiting and harmless infections. Despite the development of guidelines and risk prediction tools, variations in practice are still substantial in the management of children with suspected infection. Although some variation in practice can be explained by differences between patients, unwanted variation also occurs due to differences in practice between physicians which may lead to unnecessary testing. Therefore, this thesis aimed to describe challenges in diagnosis of children with suspected infection, and explore opportunities to improve current management in the emergency department. Part I evaluated challenges of recognition and sample collection specifically from the perspective of congenital syphilis, neonatal herpes simplex virus (HSV) infection, and pediatric severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. From the perspective of children presenting with fever without a source (FWS), part II of this thesis explored challenges of FWS guidelines through evaluation of adherence to the current Dutch national guideline and its clinical outcomes. The Dutch guideline definitions and recommendations were additionally compared to national and regional guidelines for children with FWS from other high-income countries.

PART I
CHALLENGES AND OPPORTUNITIES IN PEDIATRIC INFECTIONS

A broad spectrum of clinical manifestations, particularly in rare infections, poses challenges for recognition and leads to delay in diagnosis. **Chapter 2** described a case report of a child with unrecognized severe congenital syphilis and provided a review of diagnosis and treatment. This three-month-old boy presented with a subfebrile temperature, a nonspecific rash, portal hypertension and severe anemia. Despite being evaluated by many specialized physicians, the patient was only retrospectively diagnosed many months after discharge. Challenges of recognition in this chapter included the nonspecific clinical presentation mimicking many other diseases. Several challenges of sample collection and missed opportunities for early diagnosis were identified in this case as well, since microscopic evaluation of lesions had missed the diagnosis and a negative antenatal syphilis screening should not have ruled out the diagnosis. Although skin samples were evaluated twice in the disease course, the syphilis spirochetes were not identified because a syphilis infection was not considered and no specific staining was performed. As this case report additionally illustrated, intercurrent transmission of *T. pallidum* between antenatal screening and childbirth can still occur, even in a mother without risk factors for a syphilis infection. Thus, a diagnosis of congenital syphilis needs to be considered in any diagnostically challenging case of suspected infection, even in the context of a negative microscopic skin sample evaluation and a negative antenatal syphilis screening.

Detecting diagnostic challenges in severe, yet rare infections is important for identifying opportunities to improve diagnosis and assisting guideline development. In **chapter 3** we conducted a retrospective case series of neonates with a confirmed HSV infection in the Netherlands. Mortality and morbidity rates were very high. The clinical presentation in these cases showed substantial variation, which posed an important challenge for physicians to recognize the diagnosis. Most patients with central nervous system disease did not present with neurological symptoms and only half of patients presented with skin lesions. Furthermore, we found substantial practice variation in the type of samples collected for PCR testing and in empirical treatment. Even in the patients presenting with skin lesions there was considerable variation in sample collection. There was a treatment delay of two or more days in most patients. These results indicate opportunities for improved recognition of neonatal HSV infections and a need for national guideline recommendations for more consistent diagnostic testing.

Viral infections that are generally mild in children can, in rare cases, develop into a more severe disease course requiring hospital admission. In **chapter 4** we explored the recognition of a severe disease course in pediatric SARS-CoV-2 infection by evaluating its association with pre-existing comorbidities. Clinical characteristics and disease severity were evaluated in pediatric in- and outpatients with a SARS-CoV-2 infection in a tertiary hospital in the Netherlands, of which a little over half had a pre-existing comorbidity. Most patients, both with and without a comorbidity, presented with a mild infection. Comorbidities in children were significantly associated with the severity of infection. Particularly children with a neurological disease were found to be more prone to severe

SARS-CoV-2 infection requiring hospital admission. Children with comorbidities do not have more severe manifestations of multisystem inflammatory syndrome in children. As the confidence intervals are wide, the precise estimated effect of comorbidity remains an objective for further research.

Children have not been adequately represented in SARS-CoV-2 immunosurveillance, while they play an important role in transmission. Further, immunosurveillance is of value for estimating epidemiology and establishing effective public health measures. In **chapter 5** we evaluated the use of saliva-based assays to detect SARS-CoV-2 specific antibody prevalence compared to serum. We explored a non-invasive sampling method for detecting antibody-mediated immunity and described epidemiology among children attending medical services in the Netherlands during the first year of the pandemic. SARS-CoV-2 specific IgG in saliva showed similar antibody prevalence compared to IgG in serum. Interestingly, there was heterogeneity between the systemic and mucosal immune responses, with some children showing increased serum and saliva IgG while others were only positive in one of the two compartments (systemic or mucosal). Heterogeneity was also detected between antibodies targeting different antigens, suggesting that single-antigen based assays are to be used with care. **Chapter 6** aimed to build on the findings of **chapter 5** by evaluating the serum and saliva SARS-CoV-2 specific antibody prevalence in a higher prevalence setting than our first prevalence study. As previous studies in adults reported inconsistent results on induction and durability of systemic and mucosal SARS-CoV-2 antibodies, we additionally aimed to explore associations of antibody prevalence with demographic and clinical variables. Most children with SARS-CoV-2 specific IgG in serum were found to be detectable with our saliva-based assay. A higher correspondence between serum and saliva positivity was found after previous vaccination compared to previous infection. Moreover, sex and immunocompromization affect SARS-CoV-2 antibody prevalence in serum and saliva of children. Tracking humoral immunity through saliva-based assays could be useful for identifying SARS-CoV-2 naive populations and vaccine responses.

PART II
CHALLENGES AND OPPORTUNITIES IN FEVER WITHOUT A SOURCE GUIDELINES

Evaluating the use and impact of guideline recommendations is a crucial step in guideline improvement. The national guideline for children with FWS in the Netherlands, adapted from the National Institute for Health and Care Excellence guideline, was published by the Dutch Association of Pediatrics in 2013. In two secondary care hospitals, **chapter 7** retrospectively evaluated adherence and predictors of non-adherence to the Dutch guideline in 231 children younger than three months presenting with FWS. We detected adherence to the guideline in approximately half of patients, with even lower adherence in children younger than one month compared to children aged one to three months. Guideline recommendations that were not followed mostly included microbiological cultures or empirical antibiotic treatment, and an abnormal white blood cell count alone was not considered as an indicator to perform a full workup for a severe infection. Importantly, the clinical outcomes, including the number of missed severe infections with delayed antimicrobial treatment, were similar in the adherence and the non-adherence group. This indicates opportunities to safely do less when managing children with FWS in the emergency department. In **chapter 8** we aimed to address the objectives of **chapter 7** covering all age groups of the national guideline in a prospective regional study in seven secondary and tertiary care hospitals. In this preliminary analysis of the study data, we equally measured non-adherence in half of all children presenting with FWS. In 16% a mild viral infection was identified through rapid diagnostic testing, while no concurrent bacterial infections were found. Patients in the non-adherence group received less diagnostic testing with no difference in clinical outcomes including missed severe infections. Particularly in children younger than one month and with a high risk of severe infection according to the national guideline, physicians have used less diagnostic resources than the guideline recommended with no negative effects on clinical outcomes.

Non-adherence to guidelines and variations in practice can be explained by several factors, including inconsistency in definitions and recommendations between existing guidelines. **Chapter 9** compared the definitions and recommendations published in national and regional FWS guidelines of five high-income countries. The defined

targeted age range of children with FWS varied widely, as did the definitions for high risk criteria of severe infection. The diagnostic and treatment recommendations were broadly consistent, especially for children younger than one month. Regarding differences between recommendations, there was particular disagreement in when to perform basic diagnostic testing in children aged above three months, whereas in children aged one to three months guidelines were inconsistent in when to perform a sepsis work-up. In the context of considerable variation in current practice and 50% guideline adherence, our results imply a need for consistent, effective and practical recommendations for children with FWS. International consensus in age range, definition and management of FWS could improve future guideline development and research efforts.

Nederlandse samenvatting

De Puzzel van Kinderinfectieziekten

Uitdagingen en mogelijkheden voor het verbeteren van de diagnose in kinderen met een vermoedelijke infectie

De puzzel van kinderinfectieziekten is het onderscheid maken tussen aan de ene kant weinig frequent voorkomende ernstige infecties met mogelijk levensbedreigende gevolgen en aan de andere kant de frequente, vanzelf overgaande en onschadelijke infecties. Ondanks de ontwikkeling van richtlijnen en instrumenten voor het voorspellen van risico op een ernstige infectie, is er in de praktijk nog steeds een grote variatie in de beoordeling van kinderen met een vermoedelijke infectie. Hoewel enige variatie in de praktijk kan worden verklaard door verschillen tussen patiënten, kan er ook ongewenste variatie ontstaan door verschillen in de aanpak tussen artsen. Het doel van dit proefschrift was om uitdagingen in de diagnose van vermoedelijke kinderinfectieziekten te beschrijven, en tevens om mogelijkheden te verkennen om de huidige beoordeling van deze patiënten op de spoedeisende hulp te verbeteren. Deel I evalueerde uitdagingen in herkenning en monsterverzameling specifiek vanuit het perspectief van congenitale syfilis, neonatale herpessimplexvirus (HSV-) infecties en pediatrische severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infecties. Deel II van dit proefschrift onderzocht de uitdagingen in klinische richtlijnen vanuit het perspectief van kinderen met koorts zonder focus (FWS), door de naleving van de Nederlandse FWS-richtlijn en de klinische uitkomsten daarvan te evalueren. Daarnaast zijn de Nederlandse richtlijn definities en aanbevelingen vergeleken met nationale en regionale FWS-richtlijnen uit andere hoge-inkomenslanden.

DEEL I
UITDAGINGEN EN MOGELIJKHEDEN IN DE DIAGNOSE VAN KINDERINFECTIEZIEKTEN

Het brede spectrum van klinische manifestaties vormt uitdagingen in herkenning van kinderinfectieziekten en leidt tot vertraging in de diagnose en behandeling, met name bij zeldzame infecties. **Hoofdstuk 2** beschrijft een casus van een kind met niet-herkende ernstige congenitale syfilis en geeft een overzicht van diagnose en behandeling. Deze drie-maanden-oude jongen presenteerde zich met een subfebriele temperatuur, een aspecifieke uitslag, portale hypertensie en ernstige bloedarmoede. Ondanks dat de patiënt werd beoordeeld door vele gespecialiseerde artsen, is de diagnose pas vele maanden na ontslag achteraf gesteld. Een uitdaging in de herkenning van de diagnose uit dit hoofdstuk was bijvoorbeeld de aspecifieke klinische presentatie die ook op veel andere ziekten kan duiden. Tevens werden in deze casus verschillende uitdagingen geïdentificeerd in monsterverzameling wat leidde tot gemiste kansen voor eerdere diagnose: de microscopische evaluatie van huidmonsters had de diagnose gemist en ook had een negatieve prenatale syfilisscreening de diagnose niet mogen uitsluiten. Hoewel huidmonsters tweemaal werden geëvalueerd in het ziekteverloop, zijn de syfilis spirocheten niet geïdentificeerd omdat de diagnose niet werd overwogen en er geen syfilis specifieke kleuring werd uitgevoerd. Zoals dit rapport ook illustreerde, kan overdracht van *Treponema pallidum* nog steeds voorkomen tussen prenatale screening en bevalling, zelfs bij een moeder zonder risicofactoren voor een syfilisinfectie. Daarom concludeerden wij dat een diagnose van congenitale syfilis moet worden overwogen in elke diagnostisch uitdagende casus in infectieziekten, zelfs in de context van een negatieve microscopische evaluatie van huidmonsters zonder specifieke kleuring en een negatieve prenatale syfilisscreening.

Het detecteren van diagnostische uitdagingen in ernstige, maar zeldzame infecties is belangrijk voor de identificatie van mogelijkheden om de diagnose te verbeteren en om de ontwikkeling van richtlijnen te ondersteunen. **Hoofdstuk 3** beschrijft een reeks van neonaten met een bevestigde HSV-infectie in Nederland op retrospectieve wijze. De mortaliteit en morbiditeit in deze patiëntengroep was zeer hoog. De klinische presentatie van deze patiënten vertoonde aanzienlijke variatie, wat een belangrijke uitdaging vormde voor artsen om de diagnose te stellen. De meeste patiënten met een HSV-infectie in het centrale zenuwstelsel vertoonden aanvankelijk geen neurologische symptomen en slechts de helft van de patiënten vertoonde huidlaesies. Verder vonden we aanzienlijke praktijkvariatie in het type monsters dat werd verzameld voor PCR-testen en in de empirische behandeling. Zelfs bij de patiënten die zich presenteerden met huidlaesies was er een aanzienlijke variatie in het verzamelen van monsters. Bij de meeste patiënten was er een vertraging in de behandeling van twee of meer dagen. Deze resultaten duiden op mogelijkheden voor verbeterde herkenning van neonatale HSV-infecties en op een behoefte aan nationale aanbevelingen voor consistentere diagnostische tests.

Virale infecties die over het algemeen mild verlopen bij kinderen, kunnen zich in zeldzame gevallen ontwikkelen tot een ernstiger ziekteverloop dat een ziekenhuisopname vereist. **Hoofdstuk 4** beschrijft ons onderzoek naar de herkenning van een ernstig ziekteverloop bij pediatrische SARS-CoV-2-infecties en de associatie van ziekte ernst met comorbiditeit. Klinische kenmerken en ernst van de ziekte werden geëvalueerd bij pediatrische klinische en poliklinische patiënten met een SARS-CoV-2-infectie in een tertiair ziekenhuis in Nederland. Bij iets meer dan de helft van de patiënten werd een reeds bestaande comorbiditeit gerapporteerd. De meeste patiënten, zowel met als zonder comorbiditeit, presenteerden zich met een milde infectie. Comorbiditeit bij kinderen was significant geassocieerd met de ernst van een SARS-CoV-2 infectie. Vooral kinderen met een neurologische aandoening bleken vatbaarder voor ernstige SARS-CoV-2-infecties waarvoor ziekenhuisopname noodzakelijk was. Kinderen met een comorbiditeit hebben geen hogere kans op ernstige manifestaties van het multisysteem-inflammatoir syndroom bij kinderen na SARS-CoV-2 infectie. Omdat de betrouwbaarheidsintervallen van de associaties breed waren, blijft het precieze geschatte effect van comorbiditeit op ziekte ernst een doel voor verder onderzoek.

Kinderen zijn onvoldoende vertegenwoordigd in SARS-CoV-2 antistofrespons monitoring, hoewel ze een belangrijke rol spelen bij de overdracht van het virus. Verder is antistofrespons monitoring van waarde voor het inschatten van de epidemiologie en het vaststellen van effectieve gezondheidsmaatregelen. **Hoofdstuk 5** beschrijft de evaluatie van het gebruik van speekselmonsters om de prevalentie van SARS-CoV-2-specifieke antistoffen te detecteren in vergelijking met serum. We onderzochten een niet-invasieve methode voor monsterafname voor het detecteren van SARS-CoV-2 specifieke IgG antistoffen en beschreven de prevalentie bij kinderen in reguliere zorg in Nederland in het eerste jaar van de pandemie. SARS-CoV-2 specifiek IgG in speeksel vertoonde een vergelijkbare prevalentie in vergelijking met IgG in serum. Interessant is dat we heterogeniteit vonden tussen de systemische en mucosale immuunrespons, waarbij sommige kinderen zowel een verhoogd serum- als speeksel-IgG vertoonden, terwijl anderen alleen positief waren in een van de twee compartimenten (systemisch of mucosaal). Heterogeniteit werd ook gedetecteerd in de gemeten antistoffen gericht op verschillende antigenen, wat suggereerde dat antistoftesten gericht op een enkel antigeen met zorg moeten worden geïnterpreteerd. **Hoofdstuk 6** bouwt voort op de bevindingen van **hoofdstuk 5** door de prevalentie van SARS-CoV-2 specifiek serum- en speeksel IgG te evalueren in een setting met een hogere prevalentie dan onze eerste prevalentiestudie. Omdat eerdere onderzoeken bij volwassenen inconsistente resultaten rapporteerden over het opwekken en meetbaar blijven van systemische en mucosale SARS-CoV-2-antistoffen, wilden we de associaties onderzoeken van antistofprevalentie met demografische en klinische variabelen. De meeste kinderen met SARS-CoV-2-specifiek IgG in serum bleken ook meetbaar met onze speekseltesten. In vergelijking met een eerdere infectie werd er een hogere overeenkomst tussen serum- en speeksel IgG gevonden na een SARS-CoV-2 vaccinatie. Bovendien wordt de antistofprevalentie in serum en speeksel van kinderen beïnvloed door geslacht en een immuun gecompromitteerde status. Het meten van de antistof-gemedieerde immuniteit door middel van speekselmonsters kan nuttig zijn voor het niet-invasief identificeren van SARS-CoV-2-kwetsbare populaties en van de vaccinatieresponse.

DEEL II
UITDAGINGEN EN MOGELIJKHEDEN IN KOORTS ZONDER FOCUS-RICHTLIJNEN

Het evalueren van richtlijnen en hun impact is een cruciale stap in richtlijnverbetering. De landelijke richtlijn voor kinderen met FWS in Nederland, afgeleid van de National Institute for Health and Care Excellence-richtlijn, werd in 2013 gepubliceerd door de Nederlandse Vereniging voor Kindergeneeskunde. **Hoofdstuk** 7 evalueert retrospectief de naleving van de Nederlandse richtlijn en evalueert voorspellers van de naleving bij 231 kinderen jonger dan drie maanden met FWS in twee tweedelijns ziekenhuizen. De richtlijn werd nageleefd bij ongeveer de helft van de patiënten, met een nog lagere mate van naleving bij kinderen jonger dan een maand in vergelijking met kinderen van één tot drie maanden. Aanbevelingen uit de richtlijn die vaak niet werden gevolgd, betroffen meestal de afname van microbiologische kweken of de empirische antibioticabehandeling. Daarnaast werd een abnormaal aantal witte bloedcellen zonder andere alarmsignalen niet beschouwd als een indicator voor een volledige work-up voor een ernstige infectie. Belangrijk is dat de klinische uitkomsten, waaronder het aantal gemiste

ernstige infecties met uitgestelde antimicrobiële behandeling, vergelijkbaar waren bij naleving en bij afwijken van de richtlijn. Dit biedt mogelijkheden om veilig minder te doen tijdens de beoordeling van kinderen met FWS op de spoedeisende hulp. **Hoofdstuk 8** evalueert de doelstellingen van **hoofdstuk 7** voor alle leeftijdsgroepen van de landelijke richtlijn in een prospectieve regionale studie uitgevoerd in zeven tweede- en derdelijnsziekenhuizen. In deze voorlopige analyse van de onderzoeksgegevens vonden we wederom (net als bij de retrospectieve studie) een naleving van de richtlijn bij slechts de helft van alle kinderen met FWS. Bij 16% werd een milde virale infectie vastgesteld door middel van een diagnostische sneltest, terwijl er geen bacteriële co-infecties werden gevonden. Indien er werd afgeweken van de richtlijn werden patiënten doorgaans minder getest zonder verschil in klinische uitkomsten, waaronder gemiste ernstige infecties. Met name bij kinderen jonger dan een maand en met een hoog risico op ernstige infectie volgens de landelijke richtlijn, hebben artsen minder diagnostische middelen gebruikt dan de richtlijn had aanbevolen zonder negatieve effecten op de klinische uitkomsten.

Afwijken van richtlijnen en praktijkvariatie kunnen worden verklaard door verschillende factoren, waaronder inconsistentie in definities en aanbevelingen tussen bestaande richtlijnen. **Hoofdstuk 9** vergelijkt de definities en aanbevelingen gepubliceerd in nationale en regionale FWS-richtlijnen van vijf hoge-inkomenslanden. De definities in leeftijdsbereik van de richtlijn varieerden sterk, evenals de definities voor hoog-risicocriteria voor ernstige infectie. De aanbevelingen voor diagnosticeren en behandelen waren in grote lijnen consistent, vooral voor kinderen jonger dan een maand. Wat de verschillen tussen de richtlijnen betreft, was er met name inconsistentie in wanneer diagnostische bloedtesten geïndiceerd zijn bij kinderen ouder dan drie maanden, terwijl bij kinderen van één tot drie maanden de richtlijnen inconsistent waren over wanneer een sepsis workup moet worden uitgevoerd. In de context van aanzienlijke praktijkvariatie en matige naleving van richtlijnen, impliceren onze resultaten een behoefte aan consistente, effectieve en praktische aanbevelingen voor kinderen met FWS. Internationale consensus over de leeftijdscategorie, definitie en beoordeling van FWS zou de toekomstige ontwikkeling van richtlijnen en het onderzoek daarnaar kunnen verbeteren.

Chapter XII

Appendices

List of publications
Authors and affiliations
Authors' contributions
Portfolio
Over de auteur
Dankwoord

LIST OF PUBLICATIONS

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AUTHORS' CONTRIBUTIONS

Chapter 2 Congenital syphilis, the great imitator – a case report and review

Contributions

Study design: MK, GK, DP. Data collection: MK, GK. Writing manuscript: MK. Revision of manuscript: MK, GK, JWDZ, DSM, JvZ. Approval of manuscript: all authors. Supervision: DP.

Chapter 3 Inconsistent management of neonatal herpes simplex virus infections

Contributions

Study design: MK, DP. Data collection: MK, MvdK, JvH. Data analysis: MK. Writing manuscript: MK. Revision of manuscript: MK, MvdK, JvH, DP. Approval of manuscript: all authors. Supervision: DP

Chapter 4 Comorbidities, clinical characteristics and outcomes of COVID-19 in pediatric patients in a tertiary medical center in the Netherlands

Contributions

Study design: AB, MK, DP. Data collection: AB, KW. Data analysis: AB, MK. Writing manuscript: AB. Revision of manuscript: AB, MK, KW, DP. Approval of manuscript: all authors. Supervision: DP, MK.

Chapter 5 Saliva SARS-CoV-2 antibody prevalence in children

Contributions

Study design: MK, MB, SC, DP. Data collection: MK, AEG, EBdJ, MB, MF, FdG, DM, NO, MR. Sample processing: HvE, GK. Sample analysis: MG, KvdS, MO, RV, FL, MS. Data analysis: MK, MG, MB. Visualization: MK, MG. Writing manuscript: MK, MG. Revision of manuscript: MK, MG, MB, MR, MS, GV, TR, FP, MvG, DP. Approval of manuscript: all authors. Supervision: MvG, DP.

Chapter 6 Differences in systemic and mucosal SARS-CoV-2 antibody prevalence in a prospective cohort of Dutch children

Contributions

Study design: MK, MB, MvG, DP. Data collection: MK, MB, BA, EBdJ, MF, AEG, FdG, MR. Sample processing: HvE. Sample analysis: MG, KT, JvR, MS. Data analysis: MK, MG, MB. Visualization: MK, MG. Writing manuscript: MK, MG. Revision of manuscript: MK, MG, MB, MS, FP, MvG, DP. Approval of manuscript: all authors. Supervision: MvG, DP.

Chapter 7 Fever without an apparent source in young infants: A multicenter retrospective evaluation of adherence to the Dutch guidelines

Contributions

Study design: NK, MK, FP. Data collection: NK, JH, FP. Data analysis: NK. Writing manuscript: NK. Revision of manuscript: NK, MK, JH, DP, FP. Approval of manuscript: all authors. Supervision: MK, FP.

Chapter 8 Prospective evaluation of adherence to the Dutch guideline for children aged 0 – 16 years with fever without a source – preliminary analysis

Contributions

Study design: MK, NK, FP. Data collection: MK, NK, MB, HB, AB, MD, JH, NH, MM, AN, CN, EO, MR, MR, EvR, SvdS, SS, PT, AMvW, JvdZ, RZ. Data analysis: MK, NK. Writing manuscript: MK. Revision of manuscript: MK, NK, DP, FP. Supervision: FP.

Chapter 9 Fever without a source in children: International comparison of guidelines

Contributions

Study design: SG, MK, FP. Data collection: SG. Data analysis: SG, MK. Writing manuscript: SG, MK. Revision of manuscript: SG, MK, DP, FP. Approval of manuscript: all authors. Supervision: FP.

PORTFOLIO

Name PhD student	Maya Wietske Keuning
PhD period	January 2020 – August 2022
Name PhD supervisors	prof. dr. Dasja Pajkrt, prof. dr. Frans Berend Plötz
Name PhD co-supervisor	prof. dr. Johannes Bernard van Goudoever

PhD training

	Year	Workload (ECTS)
General courses		
BROK ('Basiscursus Regelgeving Klinisch Onderzoek')	2020	1.5
Project management	2020	0.6
Practical Biostatistics	2021	1.0
Specific courses		
Clinical Epidemiologie - Observational studies	2021	1.0
Seminars, workshops and master classes		
Weekly research meeting at Amsterdam UMC	2020- 2022	3.0
Weekly Literature club: Clinical & Laboratory Virology AUMC	2020-2022	2.0
Castor Electronic Data Capture workshop	2021	0.2
Amsterdam Kindersymposium: Masterclass in presenting	2021	0.4
VvE Webinar serie COVID-19: Modeling data in COVID 19 time	2021	0.1
WSPID Webinar: Impact of COVID-19 in the pediatric population	2021	0.1
WSPID Webinar: Schools in the COVID-19 era - to be or not to be?	2021	0.1
Presentations		
Antibiotica is een veilig alternatief voor het mes bij simpele appendicitis. Symposium presenter, Nederlandse Vereniging voor Kindergeneeskunde congres, Arnhem, the Netherlands	2019	0.5
Congenital syphilis, the great imitator. E-poster presentation, 38th Annual Meeting of the European Society of Paediatric Disease, Rotterdam, the Netherlands	2020	0.3
Inconsistent management of neonatal herpes simplex virus infections. E-poster presentation, 38th Annual Meeting of the European Society of Paediatric Disease, Rotterdam virtual edition, the Netherlands	2020	0.3
SARS-CoV-2 saliva antibodies in children. Pitch top 13 abstracts, Amsterdam Kindersymposium, Amsterdam virtual edition, the Netherlands	2020	0.3
De NVK richtlijn UWI 2019: What's new en waarom? Symposium presenter, Nier op Schier, Schiermonnikoog virtual edition, the Netherlands	2021	1.0
COVID KIDS studie - SARS-CoV-2 antistof prevalentie. Symposium presenter, Nederlandse Vereniging voor Kindergeneeskunde congres, Arnhem virtual edition, the Netherlands	2021	0.5
Saliva SARS-CoV-2 antibody prevalence in children. Pitch presentation, Nederlandse Vereniging voor Kindergeneeskunde congres, Arnhem virtual edition, the Netherlands	2021	0.5

Saliva SARS-CoV-2 antibody prevalence in children. Symposium presenter, 39th Annual Meeting of the European Society of Paediatric Disease, Geneva virtual edition, Switzerland	2021	0.5
Systemic and mucosal SARS-CoV-2 antibodies in children. Pitch presentation, Amsterdam Kindersymposium, Amsterdam, the Netherlands	2022	0.5
Are pediatric patients with comorbidities more likely to have severe COVID-19? Poster presentation. 40th Annual Meeting of the European Society of Paediatric Disease, Athens, Greece	2022	0.3
SARS-CoV-2 saliva antibodies in children. Symposium presenter. 40th Annual Meeting of the European Society of Paediatric Disease, Athens, Greece	2022	0.5

(Inter)national conferences

Nederlandse Vereniging voor Kindergeneeskunde congres, Arnhem, the Netherlands	2019	0.5
38th Annual Meeting of the European Society of Paediatric Disease, Rotterdam virtual edition, the Netherlands	2020	1.0
Amsterdam Kindersymposium, Amsterdam virtual edition, the Netherlands	2021	0.5
Nier Op Schier, Schiermonnikoog virtual edition, the Netherlands	2021	0.5
Nederlandse Vereniging voor Kindergeneeskunde congres, Arnhem virtual edition, the Netherlands	2021	0.5
39th Annual Meeting of the European Society of Paediatric Disease, Geneva virtual edition, Switzerland	2021	1.0
Amsterdam Kindersymposium, Amsterdam, the Netherlands	2022	0.5
40th Annual Meeting of the European Society of Paediatric Disease, Athens, Greece	2022	1.0

Other

Guest speaker Podcast 3x7 bij de dokter: Mijn kind heeft koorts!	2021	0.4
Organizing committee TULIPS Jonge Onderzoekersdag	2022	0.4

Teaching	Year	Workload (ECTS)

Lecturing

Teacher AUMC Bachelor kindergeneeskunde: DD bij dyspnoe	2021	2.0

Tutoring, Mentoring

Anne-Elise de Groen, managing data collection COVID KIDS Study	2020	1.0
Sanne Graaf, writing manuscript: Fever without a source in children: international comparison of guidelines	2021	1.0

Supervising

Nikki Klarenbeek, Medical Master thesis: Fever without an apparent source in young infants: A multicenter retrospective evaluation of adherence to the Dutch guidelines	2020	1.5
Özge Ozdemir, Medical Bachelor thesis: Vertical Transmission of SARS-CoV-2: a narrative review	2020	1.0
Amrita Biharie, Medical Master thesis: Comorbidities, clinical characteristics and outcomes of COVID-19 in pediatric patients in a tertiary medical center	2021	1.5
Nicole van Dijk, Medical Master thesis: Impact of the COVID-19 pandemic on the incidence of enterovirus and parechovirus in infants with fever without an apparent source	2021	1.5

Parameters of Esteem

	Year
Grants	
Subsidie Ondersteuning promovendusplek, Tergooi Wetenschapscommissie	2019
Subsidie Ondersteuning promovendusplek, Tergooi Wetenschapscommissie	2021
Travel grant, European Society of Pediatric Infectious Diseases	2022
Awards and Prizes	
Casuspresentatie: De aap uit de mouw. First place, presentation competition, Kindergeneeskunde Interklinische avond OOR Amsterdam	2019

OVER DE AUTEUR

In het portfolio op de voorgaande pagina's is te lezen wat Maya professioneel heeft gedaan tijdens haar promotietraject. Hieronder volgt een wat meer persoonlijke beschrijving van Maya, geschreven door haar moeder Tiwi en vriendinnen Anna en Roosmarijn.

Maya Wietske Keuning werd geboren op 3 januari 1992 en groeide op in een warm nest van twee culturen, Nederlands en Indonesisch, in een oud grachtenpand in hartje Amsterdam. Volgens haar moeder wilde Maya als kind al alles volgens het boekje doen, en nog beter. Dat zag je al in hoe ze haar kleurplaat inkleurde: geen plekje overslaan en door tot de lijn. Op haar 12e verloor ze haar vader aan kanker, wat grote impact heeft gehad op het gezin. Gelukkig was en is zij heel hecht met moeder, zus en even later ook Marcel.

Maya volgde haar gymnasiumopleiding op het Amsterdamse St. Nicolaaslyceum. Als klein kind niet zo fanatiek met sport maar langzamerhand ontstond toch een passie voor tennis, skiën en surfen. Van haar politiek geëngageerde opa kreeg Maya wekelijkse lessen Indonesische taal en cultuur in de Bijlmer, onder genot van de hapjes van haar oma. In 2010 begon ze met Geneeskunde aan de Universiteit van Amsterdam, geïnspireerd door haar moeder die dezelfde studie had gevolgd in China. Hard studeren werd afgewisseld met een gezellig sociaal leven en mooie ervaringen op coschap in Malawi en op reis in Indonesië.

Na haar studie ging Maya aan de slag als arts-onderzoeker bij de Federatie Medisch Specialisten om bij te dragen aan de NvK richtlijn Urineweginfecties bij kinderen. De toevoeging van een arts-assistent aan richtlijnontwikkeling was nieuw en heeft vanwege het succes geleid tot een blijvend veranderde aanpak bij richtlijnontwikkeling. Haar ervaring bij de richtlijn en haar masterscriptie over neonatale herpes bij Dasja Pajkrt waren uiteindelijk de aanzet tot dit promotietraject. Hiervoor hebben Maya en haar promotoren zelf subsidie rondgekregen o.a. via het Tergooi MC, waar ze ook met veel plezier als arts-assistent kindergeneeskunde heeft gewerkt.

Hard werken wordt nog steeds afgewisseld met een gezellig sociaal leven; haar vrije tijd brengt ze graag door met haar vriend Marinus, familie en vrienden. Ze is inmiddels een echte 'sporty spice' en doet aan (kite) surfen, boulderen en tennis. Ook houdt Maya van klaverjassen – liefst Amsterdams – , van koken – liefst Indonesisch – en van reizen – liefst gecombineerd met surfen. Binnenkort is ze dan ook de trotse eigenaar van een camperbus waarmee ze alle surfstranden van Europa kan gaan ontdekken.

DANKWOORD

De strik had nooit met dit tempo om mijn proefschrift gezeten zonder de inhoudelijke, logistieke, statistische, creatieve, culinaire of mentale ondersteuning van en samenwerking met velen. Ook al is een PhD nogal een soloproject, je komt er echt niet in je eentje. Dit hoofdstuk is een ode aan die mensen.

Allereerst mijn dank aan alle **patiënten** wiens data, spuug, bloed etc, we mochten gebruiken. Ook in tijden van corona was de bereidheid om bij te dragen enorm en dat werd echt gewaardeerd!

Dasja, als supervisor van mijn masterscriptie vijf jaar geleden heb jij mij enthousiast gemaakt voor onderzoek en onder je hoede genomen. Als een lappendeken hebben we mijn proefschrift aan elkaar gebreid. Ik vind jou een visionair en een inspirator, de professor die zegt waar het op staat, buiten gebaande paden durft en denkt in mogelijkheden in plaats van in problemen. Heel veel dank voor het zoeken naar creatieve opties, voor je drijfveer achter mijn ambitie en je enorme betrokkenheid.

Frans, ik weet nog hoe wij elkaar bij de eerste ontmoeting al gevonden hadden in het enthousiasme over richtlijnen (en wat er anders kan). Toen als mijn klinische supervisor, niet veel later als mijn promotor. Inspirerend hoe jij een netwerk van kinderartsen bij elkaar hebt weten te krijgen, in een setting waarin onderzoek niet altijd bovenaan de prioriteitenlijst staat. Dank voor het bellen toen het even tegen zat, voor de complimenten, de kritische noten en de knuppels in hoenderhokken. En ik zal natuurlijk nooit vergeten: meten is weten en weten is veranderen!

Hans, veel dank voor de jaarlijkse strategische besprekingen en je adviezen. Op een punt waarop ik zoekende was gaf jij me vertrouwen dat de richting van mijn proefschrift de juiste was. Ook het aandachtig nakijken vanuit de Oostenrijkse bergen heb ik enorm gewaardeerd!

Beste promotiecommissie, **prof. dr. Wiersinga, prof. dr. van Rossum, prof. dr. Tutu van Furth, prof. dr. Zaaijer, prof. dr. Kremer** en **dr. ir. Op de Coul**, heel veel dank! Ik voel me vereerd dat jullie de tijd en aandacht wilden investeren om jullie te buigen over mijn ideeën.

Beste **co-auteurs**, dank voor jullie input en visie op de hoofdstukken van dit proefschrift. Met in het bijzonder: het **COVID KIDS studieteam**, dankzij jullie enthousiasme en prettige samenwerking was deze logistiek in no-time opgezet. Het **FINCH studieteam**, ik ben zo trots dat we met onze resultaten impact kunnen hebben op de richtlijn en dat had niet gekund zonder jullie. **Marloes**, wat was het een fijne samenwerking en twee mooie stukken waar we heel trots op mogen zijn. Wie weet wat er nog volgt uit alle kinderspuug. Dank voor het sparren en je geduld om mij de assays uit te leggen. Veel plezier Down Under! **Hetty en Gerrit**, dank voor het meedenken, spuug aannemen en de dropjes. **Eveline**, veel dank voor het aanhoren van mijn zorgen, voor je adviezen en het meepuzzelen. **Merijn**, dank voor het statistisch en methodologisch bijsturen, dat je altijd met me mee wil denken en me ook vooral aanzet tot zelf nadenken.

Zoveel dank aan **Anne-Elise** en haar opgetuigde leger aan coassistenten en bachelor studenten: **Iris, Nina, Lisanne, Merel, Janneke, Samantha, Sarah, Anne, Dieuwke, Amrita, Benthe, Pauline, Kim, Sarah, Fruzsina, Hidde**. Zonder jullie had ik als kip zonder kop op de dagbehandeling rond gerend. Zonder jullie geen 500 inclusies vanuit het Amsterdam UMC. Jullie zijn geweldig!

Eli, zoveel dank! Op het begin voor het COVID KIDS logo waar ik altijd indruk mee maakte, en op het eind voor het prachtige eindproduct wat hier nu ligt.

Lieve **Geke en Keimpe**, veel dank! Heel gaaf dat jullie taalkundig mee wilden helpen met mijn proefschrift.

Dank je, **Zeno**, mijn lieve taal-nazi. Zonder jou was er chaos in de voorzetsels.

Mijn fijne **H8 kamergenootjes**: al hebben we een wat karige borreltijd gehad, dat gaan we met terugwerkende kracht hopelijk nog wel even goed maken!

Jason en Marleen, ESPID 2022 was geweldig! De verwachtingen waren hoog vanwege de gezelligheid in de Zoom meetings, maar Athene had dat by far overtroffen. **Julie**, zo jammer dat je pas aan kwam waaien nu de oude rotten de PIZO-club weer verlaten! Je gaat het sowieso geweldig doen.

Marre en Bart, dank voor het vertrouwen, het laten zien van mijn keuzes, en alle zetjes in de juiste richting. De energie die jullie 3-maandelijks in ons arts-assistenten stoppen is echt bijzonder!

Lieve Tergooi eetclub: **Nikki**, zonder jou geen Finch studie op dit niveau. Je gaat het vast geweldig doen in Kaapstad! **Lotte**, zo fijn om met jou tegelijk door die gekke sollicitatiemolen te gaan, dank voor je mentale support. **Mariëtte**, dank voor je adviezen in de carpoolritjes en koffieafspraken, en natuurlijk voor je 'uitgeleende spuugjes'. **Gerdine**, bedankt voor de rijsttafel, voor je steun, maar bovenal voor je creativiteit. Zonder jou was de syfilis geen winnaar en de puzzel niet compleet.

Ruim 10 jaar mijn berlinobesties **Kirsten, Arja, Zeno, Manus, Willem, Lotte, Malcolm, Vidhya, Koke, Tom, Ran**. Op nog vele jaren praten/eten/drinken/dansen/klimmen/delen/etc etc. Love you guys.

Lieve **Jip en Larry**: van 'niets van dat' in Kuala Lumpur tot Malawi avonturen, elkaar door de coschappen slepen en klagen over werk/liefdesperikelen. Ik wil jullie nooit meer kwijt, tuto bene!

Voor altijd mijn meiden: **Anna, Julia, Sterre, Roshell, Laura**. Waar iedereen ook is op de wereld, jullie voelen altijd vertrouwd. Jullie zijn familie. Zoveel zin om het kistje open te maken!

Fleddie, lieve **Anne**, mijn monyet maatje vanaf die eerste week in Bali. Met jou meer avonturen en nieuwe vrienden op je pad, meer celene bubbels in je glas en meer lol in de line-up. Dank voor je vertrouwen in mij en voor je hoe-overleef-ik-een-PhD adviezen.

Roosjematroosje, de persoon die absoluut het meeste van mij als PhD'er heeft meegekregen, die mij tijdens het schrijven van dit werk het meest heeft afgeleid en het meest heeft gesteund. De persoon met wie ik letterlijk alles zou delen. Heel graag ook de rest van het leven, samen oud worden?

Lieve **Mimi**, met jou kan ons stemgeluid naar ongekende volumes en wordt er sowieso gelachen. I love you en ik ben zo trots op je. Lieve **Stefan**, heel blij dat je bij de familie bent! Lieve **Cheyenne**, lieve **Thierry**, jullie zijn nu al de beste kids ooit en ik ben vereerd dat ik jullie tante ben.

Natuurlijk mijn ouders: lieve **mama**, matur nuwun dat je altijd voor me klaar staat (en altijd met iets lekkers). Je bent zo een sterke en zorgzame vrouw en ik heb veel respect voor hoe jij met het leven omgaat. Lieve **Marcel**, je steunt me met jouw enorme vertrouwen in mij. Ik vind jou een fantastisch en lief mens en dat jij in mijn leven bent vult een groot gemis op.

Marinus, mijn liefde, stralendste factor in mijn leven. Ook al sta jij (gelukkig) ver buiten de geneeskundebubbel, je hebt me zo vaak de (veel te directe) juiste vragen gesteld, spiegels voorgehouden en afgeleid van mijn stress met yathzee. Je bent oprecht, inspirerend en zo lief; niet voor niets iedereens lievelingspersoon. Dank je dat je me laat zijn wie ik ben, dat je hoort wanneer ik ja zeg en nee bedoel, en dat niks te gek is. Of je nou de stay-at-home dad wordt of toch die panelenworkaholic; je bent geweldig en ik hou van je.