The Diagnosis of Serious Bacterial Infections in the Children's Emergency Department

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

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Dedication

Hubert Irwin 1915-2011

Dedicated to the memory of the intrepid Hubert Irwin, with whom I travelled a long way and shared many of my happiest experiences. He taught me to see butterflies.

Abstract

Irwin AD. The Diagnosis of Serious Bacterial Infections in the Children's Emergency Department

Background

Acute febrile illness is a common presentation to the children's Emergency Department (ED). Difficulty discriminating between Serious Bacterial Infections (SBI) and self-limiting infections results in delayed treatment of SBI, and over-treatment of self-limiting infections.

Aims/Objectives

To define the aetiology of bacteraemia in the children's ED, to evaluate a universal molecular diagnostic for the diagnosis of bacteraemia, and to derive and validate risk prediction models for SBI in this setting.

Methods

A prospective diagnostic accuracy study of clinical and biomarker variables in febrile children presenting to the ED which incorporated a case-control study evaluating 16S rRNA followed by sequencing for the diagnosis of bacteraemia. An 11 year retrospective time series analysis described the aetiology of bacteraemia presenting to the children's ED. The study had full ethical approval.

Results

Time series analysis of bacteraemia presenting to the ED between 2001 and 2011 (n=575) estimated an annual 10.6% reduction in vaccine-preventable infections, and an annual 6.7% increase in Gram-negative infections. The rate of healthcare-associated bacteraemia increased from 0.18 to 0.50 per 1000 ED attendances, and the proportion of isolates susceptible to empirical antibiotics declined from 96.3% to 82.6%. Episodes of Gram-negative bacteraemia received antibiotics 1h later than episodes of vaccine-preventable bacteraemia.

1101 children were recruited to the diagnostic accuracy study. 146 children were included in an evaluation of 16S rRNA PCR in whole blood (SepsiTest) followed by sequencing for the diagnosis of bacteraemia. 120 'high-risk' children were selected alongside 26 'low-risk' children. SepsiTest identified 9/16 (56%) cases of bacteraemia. Combination with blood culture yielded a sensitivity of 75%, and specificity of 66%. SepsiTest identified 17/120 bloodstream infections with Viridans Group Streptococci in the high-risk group, and none in the low-risk group (p=0.06).

A risk prediction model combining clinical variables with the biomarkers CRP, Procalcitonin and Resistin discriminated well between Pneumonia, 'other SBIs' and no SBI (AUC 0.84 and 0.77 respectively). External validation of published models was performed and improvements in classification achieved by the addition of Procalcitonin and Resistin. The addition of biomarkers had particular value in ruling-out 'other SBIs' (NRI for non-events 5.3%).

Conclusion

Serious Bacterial Infections in the children's ED are increasingly healthcare-associated, and remain difficult to recognise. Broad-range molecular tests which are culture independent may have a role as adjuncts to conventional microbiology but require ongoing evaluation. Meanwhile, risk prediction models improve discrimination between SBI and self-limiting infections and should be tested in robust impact studies.

Publications

Published work describing results presented in this thesis:

Irwin AD, Drew RJ, Marshall P, Nguyen K, Hoyle E, Macfarlane KA, Wong HF, Mekonnen E, Hicks M, Steele T, Gerrard C, Hardiman F, McNamara PS, Diggle PJ, Carrol ED. Etiology of Childhood Bacteremia and Timely Antibiotics Administration in the Emergency Department. *Pediatrics.* 2015; 135 (4): 635-42.

Manuscripts in preparation:

Irwin AD, Grant A, Williams R, Kolamunnage-Dona R, Drew RJ, Paulus S, Jeffers G, Williams K, Breen R, Newman J, Appelbe D, Chesters C, Newland P, Marzouk O, McNamara PS, Diggle PJ, Carrol ED. Derivation, validation and updating of risk prediction models for the diagnosis of serious bacterial infections in febrile children presenting to the Emergency Department.

Irwin AD, Grant A, Williams R, Paulus S, Barton T, Jeffers G, McNamara PS, Diggle PJ, Carrol ED. Broad-range PCR and sequencing for the evaluation of febrile children presenting to the Emergency Department.

Published work relevant to, but not directly pertaining to results presented in this thesis:

Sabhaney V, Enarson P, **Irwin AD,** Carrol ED. Procalcitonin-stewardship study group. Commentaries on 'Procalcitonin to initiate or discontinue antibiotics in acute respiratory tract infections' with a response from the review authors. *Evid Based Child Health*. 2013 Jul;8(4):1372-5

Irwin AD, Marriage F, Mankhambo LA, IPD Study Group, Jeffers G, Kolamunnage-Dona R, Guiver, M, Denis B, Molyneux EM, Molyneux ME, Day PJ, Carrol ED. Novel biomarker combination improves the diagnosis of serious bacterial infections in Malawian children. *BMC Med Genomics.* 2012; 5: 13.

Irwin AD, Carrol ED. Procalcitonin. *Arch Dis Child Educ Pract Ed.* 2011; 96(6): 228-33.

List of abbreviations

ACF	Autocorrelation function
AMR	Antimicrobial resistance
BSAC	British Society for Antimicrobial Chemotherapy
CA	Community-acquired
CE	Conformité Européenne
CI	Confidence interval
CM	Significant comorbidity
CRF	Case report form
CRP	C-reactive Protein
CV	Coefficient of variation
CVL	Central Venous Line
DNA	Deoxyribonucleic acid
ED	Emergency Department
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ENT	Ear, nose, throat
FN	False negative
FP	False positive
FV	Fitted values
GAM	Generalized additive model
GN	Gram negative
HCA	Healthcare-associated
Hib	*Haemophilus influenzae type b*
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon gamma
iGAS	Invasive Group A Streptococcus
IL	Interleukin
IOS	Infant observation score
IPD	Invasive pneumococcal disease
IQR	Interquartile range

LCI	Lower confidence interval
LE	Leucocyte esterase
LOS	Length of stay
LPS	Lipopolysaccharide
LR	Likelihood ratio
MALDI-TOF	Matrix-assisted laser desorption ionisation-time of flight
MBL	Mannose Binding Lectin
MCD	Meningococcal disease
MS	Mass spectrometry
NF-κβ	Nuclear factor kappa beta
NGAL	Neutrophil Gelatinase-associated Lipocalin
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
NIHR	National Institute for Health Research
NLR	Negative likelihood ratio
NPV	Negative predictive value
OR	Odds ratio
Oth.GP	Other Gram positive
PCR	Polymerase chain reaction
PCT	Procalcitonin
PCR	Polymerase chain reaction
PCV	Pneumococcal conjugate vaccine
PICU	Paediatric Intensive Care Unit
PLR	Positive likelihood ratio
PPV	Positive predictive value
RCT	Randomised controlled trial
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RTN	Resistin
SBI	Serious bacterial infection
SE	Standard error

SPICED	Salivary Procalcitonin in the Children's Emergency Department
TGF	Transforming growth factor
TN	True negative
TNF	Tumour necrosis factor
TP	True positive
TLR	Toll-like receptor
TPN	Total parenteral nutrition
Typ.GP	Typical Gram positive
UCI	Upper confidence interval
UKCRN	United Kingdom Clinical Research Network
UMD	Universal microbe detection
VCAM-1	Vascular cell adhesion molecule-1
VP	Vaccine-preventable
WCC	White blood cell count

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Chapter 1: Introduction

1.1. Synopsis of thesis

The febrile child presents a common diagnostic dilemma. Most children presenting to the Emergency Department (ED) with an acute febrile illness will have a self-limiting viral illness. A small number, however, will have a Serious Bacterial Infection (SBI), and will benefit from the prompt administration of antibiotics. Recognition of those children with SBI is often difficult, and poor discrimination between SBI and self-limiting illnesses may result in both delayed treatment of SBI, and over-treatment of self-limiting illness. This thesis seeks to address the challenge of improving diagnostic discrimination in febrile children in the ED.

1.2. Overview of the chapter

In this Chapter I aim to describe the burden of acute febrile illness in children presenting to the ED, the importance of identifying SBI in these children, and the difficulty of doing so. I set out the current diagnostic approach to the febrile child, including the use of clinical findings and laboratory tests. With reference to the host immune response to serious infection, I describe the existing evidence for newer markers of SBI in the children's ED, and highlight the challenges associated with undertaking diagnostic accuracy studies in this field. Finally, I set out in detail the aims and objectives of the thesis.

1.3. Background

1.3.1. Acute febrile illness in the children's Emergency Department

In the UK in 2013, approximately 4 million children under the age of 16 attended the ED[1]. Acute febrile illnesses are the most common of all medical presentations to the children's ED, the majority of which are caused by acute self-limiting infections[2]. Data from a single UK ED estimated that fever as a primary reason to attend ED accounted for 14% of all childhood medical presentations, and that acute infections (of which fever may have been a part) accounted for more than one half[3]. The same

study estimated that the number of medical presentations to the children's ED increased by 40% between 2001 and 2011[3,4]. Though there are no equivalent data from the UK, recent national data from the US reveal that almost one third of all childhood presentations to the ED are infection-related, of which 59% are upper respiratory or ear infections[5].

1.3.2. Serious Bacterial Infection in the children's Emergency Department

SBI is a heterogeneous term representing a group of infections requiring prompt and definitive antimicrobial therapy. Diagnoses reported as SBI in diagnostic accuracy studies in children are listed in Table 1. In young children in highly resourced settings SBI account for an estimated 7% of acute febrile illnesses presenting to the ED[6].

Category of SBI
Bacteraemia/septicaemia
Meningitis
Pneumonia
Urinary tract infection/pyelonephritis
Osteomyelitis
Septic arthritis
Bacterial gastroenteritis
Mastoiditis

Table 1: Diagnoses reported as SBI in diagnostic accuracy studies in children[7-10]

SBI are associated with significant morbidity and mortality in children. Meningococcal disease (MCD) - septicaemia or meningitis caused by *Neisseria meningitidis* - is the most common fatal bacterial infection in UK children[11]. Whilst its incidence in the UK has declined following the introduction of the conjugate vaccine against serogroup C[12], it remains higher than that of other European

countries[13]. Delays in the treatment of MCD and other severe sepsis increase mortality, and the likelihood of long term disability[14-17].

Pneumonia and urinary tract infection (UTI) are the most common SBIs seen in the children's Emergency Department[6]. Whilst the introduction of effective vaccines against *Haemophilus influenzae* type b (Hib) and *Streptococcus pneumoniae* has reduced the contribution of these bacterial pathogens to the burden of bacterial pneumonia[18,19], complications from pneumonia remain an important cause of hospital admission in the UK[20]. Globally, pneumonia remains the single most important cause of childhood death[21].

Childhood UTI exerts a significant and increasing burden on hospital services. In the UK, hospital admission rates of children with UTI increased by 39% between 2001 and 2011[22]. UTI may be associated with septicaemia and meningitis, particularly in young infants or immunocompromised children. Approximately one third of children with UTI involving the upper urinary tract subsequently develop renal scars[23].

1.3.3. Epidemiology of Serious Bacterial Infection in children

1.3.3.1. The global burden of Serious Bacterial Infection in children

Globally, SBI remains a substantial contributor to child mortality. Pneumonia alone is estimated to cause 1.5 million deaths in children under 5 each year[21,24]. Child deaths attributable to invasive *Streptococcus pneumoniae* and *Haemophilus influenzae* type b infections are estimated to equal those collectively attributable to HIV, Tuberculosis and malaria[18,19]. In malaria endemic regions, the diagnosis of malaria frequently leads to a failure to recognise and treat SBI[25,26]. Simultaneously, World Health Organisation (WHO) guidelines for the management of childhood febrile illness in these regions encourage the use of antibiotics in often self-limiting non-malarial febrile illness[26], reflecting a failure both to recognise SBI when it is present, and to rule it out when it is absent.

'Sepsis' (which I will discuss in more detail in Section 1.6.2) describes the systemic manifestations of the inflammatory response to serious infection. Recently, a large

international study of paediatric intensive care units (PICUs) estimated the point prevalence of 'severe sepsis' to be 8.2% of all PICU patients. More than three quarters of cases of severe sepsis occurred in children with comorbidities. Mortality was approximately 25%, and 17% of survivors developed a significant disability[27]. Evidence from both North America and Australasia suggests that its frequency has increased over the last decade[28,29]

1.3.3.2. Serious Bacterial Infection in children in the UK

In the UK, SBI caused by Hib[30], *Neisseria meningitidis*[12,31,32], and *Streptococcus pneumoniae*[20,33] have declined dramatically following the introduction of effective conjugate vaccines. Other invasive bacterial pathogens for which effective immunisations have yet to be implemented continue to cause significant disease however. Outbreaks of invasive Group A Streptococcus (iGAS) infections associated with substantial mortality have been reported in the UK in recent years[34,35]. Toxic shock syndrome caused by both iGAS and *Staphylococcus aureus* is rare – with an estimated incidence of 0.38 per 100000 children in the UK – but often requires intensive care support, and carries a significant risk of death[36]. Overall, SBI are estimated to cause 20% of all child mortality in the UK[37].

The UK's NHS Hospital Episode Statistics database provides publicly available national data for all clinical episodes (http://www.hscic.gov.uk/hes). In the year to April 2013, there were approximately 39 000 clinical admissions of children under the age of 16 years old with SBI as a primary diagnosis. Figure 1 illustrates the most common SBI diagnoses requiring admission to hospital.

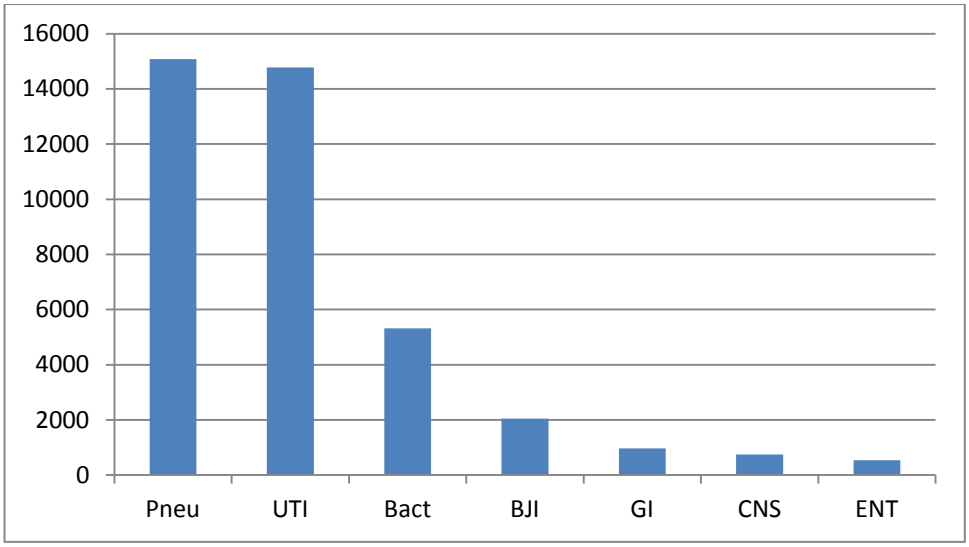


Figure 1: Number of hospital admissions in children (<16y) with SBI in the UK in 2013[38]. Pneu: Bacterial pneumonia and empyema. Bact: Bacteraemia. BJI: Bone and joint infection. GI: Bacterial enteric infection. CNS: Bacterial meningitis. ENT: Mastoiditis.

1.3.3.3. Bacteraemia in children in the UK

Bacteraemia in children in the UK is now uncommon in previously healthy children. A study of bacteraemia and meningitis in hospitals in south London between 2009 and 2011 estimated the population incidence of 'community-acquired' bacteraemia or meningitis – that is, bacteraemia and meningitis diagnosed within 48 hours of presentation to hospital – to be 18 per 100 000. Two thirds of these cases occurred in children with significant comorbidities. In children with no comorbidities, the estimated incidence of community-acquired bacteraemia was 5.3 per 100 000[39]. Low estimates of age standardised hospital admission rates for bacteraemia and meningitis caused by *N.meningitidis, S.pneumoniae* and Hib have similarly been reported in the UK, alongside longitudinal data demonstrating the impact of immunisation on declining rates[12].

Whilst the incidence of childhood bacteraemia in the UK caused by vaccine-preventable organisms has declined, a 10 year study of data from NHS microbiology databases revealed an increase in both Gram-negative infections (such as Enterobacteraeciae), and less virulent Gram-positive infections such as Coagulase negative Staphylococci and non-pyogenic Streptococci[40].

In summary, acute febrile illnesses make up a significant proportion of all presentations to the children's ED. Of these, SBI are a small but important component. Not only are SBI relatively uncommon, but the presentation of SBI is evolving as immunisation reduces the incidence of the most common invasive childhood pathogens. Furthermore, children presenting to the ED with SBI are increasingly those with significant comorbidities. Understanding changes in the aetiology of, and risk factors associated with, childhood SBI is necessary in order to improve its recognition and treatment. Improving the ability of clinicians to discriminate between SBI and self-limiting febrile illness would allow prompt intervention and therapy in those who need it, whilst supporting rational decision-making regarding antimicrobial use and the need for hospital admission.

1.4. Diagnosing Serious Bacterial Infection in children

1.4.1. The importance of prompt identification: 'Ruling in' SBI

The prompt recognition and treatment of SBI improves outcomes, and forms the basis of management in sepsis. Clinical guidelines commonly refer to the 'golden hour' of sepsis management. This is the recommendation that antibiotics are commenced within one hour of recognition of severe sepsis, based upon the demonstration of increasing mortality for every hour of delayed therapy[41].

In children with suspected sepsis in the ED, average time to administration of antibiotics is more than 2 hours from arrival[42]. In the case of MCD, delays to antibiotic administration are associated with increased mortality[43], while pre-hospital antibiotics reduce the likelihood of death[44]. The signs and symptoms of MCD are non-specific early in the disease process and rapidly progressive[45]. Approximately one half of children with MCD are not identified at first contact with medical services[14,45].

Prompt and appropriate antimicrobial therapy for other SBIs is intuitively important. Though there is little evidence to associate delayed antibiotics with adverse outcomes in children with pneumonia in the ED, a small study of children in

paediatric intensive care reported that longer time to appropriate antibiotics was associated with an increased duration of mechanical ventilation [46]. Delays in the treatment of UTI in infants have been associated with the development of subsequent renal scarring[47].

1.4.2. Rational diagnosis: 'Ruling out' SBI

The consequences of poor diagnostic discrimination are not limited to a failure to 'rule in' serious infection. Faced with diagnostic uncertainty, and difficulty 'ruling out' serious infection in the ED, clinicians are committed to prolonged clinical observation, including the possibility of unnecessary admission to hospital and antimicrobial treatment. For those children discharged home, appropriate strategies to allow the recognition of a deteriorating condition ('safety-netting') are necessary[48].

An increase in short-stay uncomplicated hospital admissions in children suggests that clinicians are adopting an increasingly risk-free approach to the febrile child[22,49]. Over the last decade, the likelihood of a young child being admitted to hospital with an acute infection categorised as 'primary care sensitive' – one which may be appropriately managed in the community – has approximately doubled[22]. An increasing burden of medical attendances to ED[3], associated with changes to medical training and working patterns[50] may all have contributed to this minimum risk approach. Improving the ability of clinicians to confidently rule out SBI has significant potential to rationalise hospital admission.

Alongside decisions relating to the need for hospital admission, diagnostic uncertainty in acute febrile illness may also result in the overuse of antibiotics. Guidelines from both the National Institute for Health and Care Excellence (NICE) and the British Thoracic Society advocate the use of antibiotics for the management of respiratory infections in children where 'the patient is systemically very unwell', or has 'signs and symptoms suggestive of serious illness'[51,52]. The subjectivity of these assessments may partly underlie the substantial geographical variation in antibiotic prescribing observed across even highly immunised populations. Large ecological studies from Europe identified a threefold variation in antibiotic

prescribing between the highest and lowest prescribing nations[53,54]. This prescribing is strongly associated with the occurrence of antibiotic resistant organisms[54]. Improving rational prescribing is a central component of the World Health Organisation global strategy for the containment of antimicrobial resistance[55].

In view of the substantial difficulty faced by clinicians in ruling in, or ruling out SBI, the value of clinical signs and adjunctive diagnostic tests has been extensively evaluated in diagnostic accuracy studies.

1.5. Methodological issues in diagnostic accuracy studies

Before summarising diagnostic accuracy studies of SBI in febrile children presenting to the ED, I will describe the importance of robust methods in the undertaking and reporting of such studies.

Diagnostic accuracy measures the agreement between the test of interest (the 'index test') and the target condition as determined by a pre-defined reference standard. The validity of the findings of diagnostic accuracy studies, and their applicability to clinical care are dependent upon the methodology applied. Poorly designed studies are associated with biased, often optimistic assessments of diagnostic performance[56,57]. Furthermore studies in which the population, test and outcome of interest are ill-defined are difficult to interpret.

1.5.1. Standards for Reporting of Diagnostic Accuracy (STARD) guidelines

Concern regarding the design and reporting of diagnostic accuracy studies prompted the Cochrane diagnostic and screening test methods working group to develop a checklist of items to be included in diagnostic accuracy studies, resulting in the Standards for Reporting of Diagnostic Accuracy (STARD, Table 2)[58]. The objectives of the STARD initiative were to improve the clear reporting of diagnostic accuracy studies in order to allow readers to evaluate potential bias in a study (which determines the internal validity of the study), as well as to assess the generalisability of the findings (that is, its external validity).

Section and Topic	Item #		On page #
TITLE/ABSTRACT/ KEYWORDS	1	Identify the article as a study of diagnostic accuracy (recommend MeSH heading 'sensitivity and specificity').	
INTRODUCTION	2	State the research questions or study aims, such as estimating diagnostic accuracy or comparing accuracy between tests or across participant groups.	
METHODS *Participants*	3	The study population: The inclusion and exclusion criteria, setting and locations where data were collected.	
	4	Participant recruitment: Was recruitment based on presenting symptoms, results from previous tests, or the fact that the participants had received the index tests or the reference standard?	
	5	Participant sampling: Was the study population a consecutive series of participants defined by the selection criteria in item 3 and 4? If not, specify how participants were further selected.	
	6	Data collection: Was data collection planned before the index test and reference standard were performed (prospective study) or after (retrospective study)?	
Test methods	7	The reference standard and its rationale.	
	8	Technical specifications of material and methods involved including how and when measurements were taken, and/or cite references for index tests and reference standard.	
	9	Definition of and rationale for the units, cut-offs and/or categories of the results of the index tests and the reference standard.	
	10	The number, training and expertise of the persons executing and reading the index tests and the reference standard.	
	11	Whether or not the readers of the index tests and reference standard were blind (masked) to the results of the other test and describe any other clinical information available to the readers.	
Statistical methods	12	Methods for calculating or comparing measures of diagnostic accuracy, and the statistical methods used to quantify uncertainty (e.g. 95% confidence intervals).	
	13	Methods for calculating test reproducibility, if done.	
RESULTS *Participants*	14	When study was performed, including beginning and end dates of recruitment.	
	15	Clinical and demographic characteristics of the study population (at least information on age, gender, spectrum of presenting symptoms).	
	16	The number of participants satisfying the criteria for inclusion who did or did not undergo the index tests and/or the reference standard; describe why participants failed to undergo either test (a flow diagram is strongly recommended).	
Test results	17	Time-interval between the index tests and the reference standard, and any treatment administered in between.	
	18	Distribution of severity of disease (define criteria) in those with the target condition; other diagnoses in participants without the target condition.	
	19	A cross tabulation of the results of the index tests (including indeterminate and missing results) by the results of the reference standard; for continuous results, the distribution of the test results by the results of the reference standard.	
	20	Any adverse events from performing the index tests or the reference standard.	
Estimates	21	Estimates of diagnostic accuracy and measures of statistical uncertainty (e.g. 95% confidence intervals).	
	22	How indeterminate results, missing data and outliers of the index tests were handled.	
	23	Estimates of variability of diagnostic accuracy between subgroups of participants, readers or centers, if done.	
	24	Estimates of test reproducibility, if done.	
DISCUSSION	25	Discuss the clinical applicability of the study findings.	

Table 2: Standards for Reporting of Diagnostic Accuracy (STARD) checklist. http://www.stard-statement.org. Accessed 11th December 2013.

1.5.2. Evaluating diagnostic accuracy in the absence of a 'gold' standard

A significant methodological challenge in diagnostic accuracy studies is that of establishing appropriate reference standards against which to evaluate the index test of interest. A 'gold' reference standard would establish the presence or absence of the target condition, reliably identifying the true state of each study subject. In the context of infectious disease diagnostics, few such 'gold' standards exist. Reference standards based upon microbiological confirmation of infection are limited by their own lack of sensitivity[59]. In children, even obtaining microbiological samples for respiratory infections[60,61], or bone and joint infections may be difficult[62]. As a result, surrogate measures such as radiological evidence of bacterial pneumonia or osteomyelitis are often used, despite their limitations[63]. This can lead to misclassification of cases which may have an impact on the estimated performance of the index test under investigation[64,65].

1.5.2.1. Misclassification

In the classical diagnostic accuracy paradigm, disagreement between index test and reference test is considered to be a 'false' result of the index test, while classification of the target condition by the reference standard is considered error free. Classification errors of the target condition by the reference standard will thus affect estimates of the accuracy of the index test. The impact of this misclassification is hard to predict. If errors of classification occur at random and are independent of the index test, then misclassification is said to be 'non-differential'. Such non-differential misclassification results in estimates of diagnostic accuracy which are biased towards the null – it under-estimates the diagnostic accuracy of the index test. Conversely, where misclassification errors are correlated with errors in the index test ('differential misclassification'), this results in erroneous agreement between the index and reference tests and inflated estimates of diagnostic accuracy[64,65].

In order to remedy the impact of imperfect reference standards on estimates of diagnostic accuracy, various approaches have been described. These have been extensively reviewed[64].

1.5.2.2. Adjustments to the reference standard

One approach to the problem of an imperfect reference standard is to adjust estimates of diagnostic accuracy of the index test based upon external data describing the accuracy of the reference standard. If reliable estimates of diagnostic accuracy of the reference standard are available, and if the error inherent in the reference standard is independent of error in the index test, then statistical corrections to the accuracy of the index test can be made to account for known error in the reference test. Often however, little is known about the 'true' accuracy of the reference standard. In this case sensitivity analyses may be undertaken which adjust for the accuracy of reference test across a range of plausible estimates. Where the assumption of conditional independence between the errors is false, a correlation parameter may be included, and if the degree of correlation is unknown this too may be varied across a range of plausible values in order to adjust estimates for the index test[66].

1.5.2.3. Composite reference standard

An alternative approach to the use of an imperfect reference standard is to seek a consensus outcome from a number of 'expert' investigators. These approaches have proved popular, but risk incorporation bias whereby index tests under investigation are used as part of the rule to determine outcome[64,65]. In this context, the incorporation of the index test may result in inflated estimates of index test accuracy. One solution to this is to use a pre-defined rule to define the composite reference standard in terms of a number of imperfect reference standards. While the use of expert panels may bring together significant expertise in order to define a reference standard, this lacks a formal data-driven methodology.

1.5.2.4. Latent class analysis

Latent class analysis is a formal statistical approach that combines multiple pieces of information in order to construct a composite reference standard. In this case, an unknown 'latent' variable (for example, the presence or absence of SBI) can be modelled against observed 'manifest' variables, including any imperfect reference

standards. The probabilities of observing true and false positive and negative test results can be described in terms of the (unknown) sensitivity and specificity of each test and the (observed) prevalence. Using maximum likelihood methods, the solution (the estimates of sensitivity and specificity for each of the latent variables) which best describes the observed data can be obtained[67].

While the use of latent class models is well established, and the statistical solution satisfying, latent class models may be criticised for failing to define the latent variable clinically - it can be hard to understand what exactly the target condition defined by the latent variable is. Furthermore, the incorporation of the index test into the definition of the target condition is inherent to the latent class method, though it could be argued that this is a less significant problem than in other methods which define a composite reference standard.

1.5.2.5. Evaluating improvements to the reference standard

When the limitations of an existing reference test are recognised, disagreement between the results of a new index and the existing reference test may represent an improvement in diagnostic accuracy. Evaluating a molecular method of infectious disease diagnosis based on PCR against a culture based reference standard with limited sensitivity, for example, may yield new positive 'cases'. These may represent false positive results or an improvement in sensitivity against the existing standard. Importantly, these new identifications may represent a new definition of the target condition. Are these new cases associated with less severe disease, and what are the consequences of their identification? Understanding the implications of discordant cases is necessary in order to determine whether the new index test is an improvement on the reference standard.

Evaluating discordance between index and reference tests may be achieved using a third test. This test, which has been called an 'umpire' test, must be related to the target condition, but independent of both the index and reference tests under evaluation. It may be a related exposure, another diagnostic test, or a response to treatment. Inevitably an imperfect diagnostic test for the outcome of interest, it only matters that its errors are unrelated to those of the other tests[68].

An example of this approach was given in the evaluation of the interferon gamma release assay, ELISPOT, for the diagnosis of latent Tuberculosis infection (LTBI) in the context of a school TB outbreak[69]. This study compared the use of ELISPOT to the established, but recognisably imperfect reference standard of the Tuberculin skin test (TST). Though there was substantial agreement between the two tests, in approximately 11% (61/535) of children, the tests were discordant. The authors used a third measure – that of exposure to the index TB case – as an independent adjudicator. In identifying that the ELISPOT correlated more closely with TB exposure, it concluded that the method was an improvement on the TST for the identification of LTBI. This assertion was tested further by the follow up of children identified to have positive and negative tests to look at subsequent development of TB disease, which in this case is the clinical condition of most consequence.

1.5.3. Diagnostic accuracy studies in paediatric SBI

Diagnostic accuracy studies in paediatric SBI present particular problems. As described above, misclassification of study subjects caused by imperfect reference standards may impact the accuracy, and internal validity of published studies. The external validity of studies meanwhile is frequently limited by restrictive inclusion criteria such as those based upon age, or temperature at presentation. These inclusion criteria may themselves be associated with the likelihood of SBI. External validity is limited too by the heterogeneity of studied populations, and by the use of various definitions of SBI. Evidence of this heterogeneity can be seen as variation between studies in the proportion of subjects with a diagnosis of SBI[8].

Finally, studies which incorporate the use of invasive tests, including simple blood tests in children, may be considered ethically justifiable only if undertaken in children already undergoing invasive clinical investigations. In the ED, this results in the study of a population of children at higher risk of SBI. Applying findings from such a population to a lower risk group may not be appropriate.

An understanding of the requirements of diagnostic accuracy studies is important both in the design of high quality studies and in their interpretation. Methodologically robust studies ensure that reliable estimates of diagnostic accuracy can be made, upon which to consider the implications for clinical practice.

1.6. Current approaches to the diagnosis of SBI in the children's ED

Here I aim to describe the current diagnostic approach to SBI in children in the ED, moving from the ideal scenario of rapid, accurate pathogen identification, to the use of indirect evidence of SBI based upon clinical or biomarker variables.

1.6.1. Pathogen identification

1.6.1.1. Culture-based methods of identification

The ideal diagnostic test for SBI would allow rapid, accurate identification of the causative organism in order to guide timely and effective antimicrobial treatment. In children, obtaining appropriate samples is the first challenge to overcome. Sampling for possible UTI is relatively straightforward, but the process of obtaining adequate samples for the diagnosis of pneumonia, meningitis and bacteraemia are all associated with significant difficulty. Sputum samples from children with suspected pneumonia require the child to be able expectorate, while the identification of an organism, particularly if from the upper respiratory tract, does not attribute causation[70]. Obtaining adequate samples of CSF and blood may also be a technically demanding, particularly in unwell children. For the diagnosis of bacteraemia the volume of blood obtained is directly associated with the culture yield[59].

Culture-based methods of pathogen identification are limited most obviously by the time they require. Following the initial identification of a positive isolate in a clinical sample, conventional pathogen identification involves a process of phenotypic testing designed to characterise the isolate[71]. This process of analysis may take

several days to complete. An alternative to this phenotypic approach uses a technique called matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry (MS). In this, a sample taken from a positive culture isolate is mixed with a matrix material before ablation using a pulsed laser. The resultant peptides are ionised and accelerated into a mass spectrometer. Identification of the organism is achieved by comparing the peptide spectrum of the sample with reference spectra[72]. Positive evaluations of MALDI-TOF MS in terms of the reliability of pathogen identification, time taken, and cost involved have encouraged uptake into clinical practice[73]. Other platforms at a more exploratory stage of development are discussed in Chapter 4.

Though techniques such as MALDI-TOF MS promise to reduce the time from positive culture to pathogen identification, culture-based methods are insufficiently timely to impact treatment decisions at the time of diagnosis.

1.6.1.2. Molecular methods of identification

Culture independent methods of pathogen identification are of substantial interest. Polymerase chain reaction (PCR) is now a well-established technique which allows the amplification of specific regions of – in this context – bacterial DNA complementary to the primers used in the assay[74] (see Section 4.2.2). PCR-based testing may be completed within a few hours. For the diagnosis of bacteraemia, the technique requires smaller volumes of blood than culture, and is less affected by prior antibiotic treatment[75]. Specific PCR assays for the identification of individual organisms, such as *N.meningitidis* are highly sensitive and specific[76], but only provide information about the presence or absence of the target organism. For the diagnostic assessment of the febrile child, the molecular approach would need to identify a broad range of potential pathogens.

In recent years, a number of broad-range molecular tests for the diagnosis of SBI have been developed and evaluated[77]. These tests are largely based upon identification and amplification of highly conserved regions of 16S ribosomal RNA (rRNA), and can be considered in two groups. 'Multiplex' PCR assays target a range of likely causative organisms using a panel of pathogen-specific probes, whilst

'universal' PCR assays achieve speciation with the use of a post-PCR process such as DNA sequencing or mass spectrometry[78]. Commercially available broad-range molecular tests are detailed in Chapter 4.

The first broad-range molecular test to achieve the European CE mark for the diagnosis of bacteraemia was SeptiFast (Roche Diagnostics, Mannheim, Germany). Pathogen identification using SeptiFast is based upon the use of species-specific probes for 25 of the most common bloodstream pathogens. *In vitro* it has been found to be highly sensitive and specific[79]. In clinical diagnostic accuracy studies, however, its performance has been more variable. A recent systematic review funded by the National Institute for Health Research (NIHR) highlighted substantial methodological weaknesses of the published evidence and failed to reach clear conclusions about its clinical utility[80].

The use of broad-range PCR assays in whole blood samples is a technical challenge. Host DNA is present in whole blood in much greater quantities than pathogen DNA, and inhibitory to the PCR reaction necessary for pathogen detection[77]. One solution to this is to selectively lyse host cells and degrade host DNA, recovering intact pathogens before isolation of pathogen DNA[81]. MolYsis (Molzym, Bremen, Germany) is a commercially available pre-analytical process designed in this way to selectively extract pathogen DNA. The method has been demonstrated to substantially reduce host DNA contamination, and increase the sensitivity of pathogen detection in clinical whole blood samples[82].

In adults, PCR amplification of variable regions of the 16S rRNA gene followed by sequence analysis (using SepsiTest, Molzym) has proven a sensitive method for detecting and identifying a broad range of aetiologies of sepsis and infectious endocarditis[83,84]. The sensitivity of this approach in small volume blood samples from children has yet to be demonstrated however.

1.6.2. Clinical indicators of SBI in children

In the absence of timely, direct pathogen identification, clinicians are required to look for indirect evidence of the presence of SBI. The systemic response to serious infection is termed 'sepsis'. More accurately, sepsis describes manifestations of a dysregulated host immune response associated with severe infection and organ dysfunction[85]. Sepsis in children was defined at an international conference in 2005[86], modifying criteria established in adults[87]. Age appropriate clinical (and biomarker) variables determine the presence of the Systemic inflammatory response syndrome (SIRS), while sepsis requires the presence of SIRS alongside evidence of a causative pathogen which may be microbiologically proven or strongly suspected on clinical grounds. As a consensus definition, the term is not without limitations. A large recent study of adult intensive care patients found that the use of SIRS criteria to determine the presence of sepsis excluded 1 in 8 patients with 'severe sepsis'[88].

Whilst primarily designed to address the challenge of sepsis in a critical care setting, the definition highlights some of the clinical features consistent with systemic infection (such as temperature, heart rate and respiratory rate) and is relevant to the diagnosis of SBI in the ED. Extensive investigation has been undertaken in the paediatric ED to identify those clinical features that delineate SBI from self-limiting infection, or illnesses of a non-infectious nature.

The value of clinical signs in the diagnosis of serious infections in children was comprehensively summarised in a recent systematic review [10]. Most of the published studies were undertaken in EDs where the likelihood of SBI was moderate (5-20%)[89-96] or high (>20%)[97,98]. Table 3 details the performance characteristics of individual clinical signs for the diagnosis of SBI in children presenting to the ED. Though a number of clinical signs were associated with the presence of SBI, few were sufficiently discriminatory to either rule in or rule out SBI. An explanation of the terms used to quantify diagnostic accuracy (such as the likelihood ratios presented in the table) is given in Section 2.6.1.

Much of the published evidence was of a modest quality. In particular the challenge of attributing outcome diagnosis in the absence of a well-defined reference standard, and issues relating to blinding of investigators to outcome diagnosis limited the value of the published evidence. Moreover, many of the studies evaluated children suspected of a specific diagnosis (such as meningitis or MCD), limiting their generalisability to the population of children presenting to the ED.

Indicator	Reference	Outcome diagnosis	Proportion with SBI (%)	Age range/ months	LR+	LR-
Ill appearance	Waskerwitz 1981[89]	Bacteraemia	5.8	0-24	2.75 (1.56 – 4.86)	0.64 (0.41 – 1.00)
	Haddon 1999[99]	Bacteraemia	3.4	3-36	1.05 (0.15 – 7.48)	1.00 (0.9 – 1.11)
	Wells 2001[95]	Bacteraemia	11.0	0-180	4.27 (2.98 – 6.11)	0.26 (0.12 – 0.56)
	Bleeker 2007[97]	All SBI	26.0	0-36	1.40 (1.15 – 1.71)	0.67 (0.50 – 0.88)
	Nijman 2013[96]	Pneumonia	14.5	1-180	1.55 (1.22 – 1.97)	0.86 (0.78 – 0.95)
	Nijman 2013[96]	Other SBI	14.5	1-180	1.68 (1.34 – 2.10)	0.83 (0.75 – 0.93)
Abnormal cry	Oostenbrink 2001[98]	Meningitis	38.7	1-180	1.34 (0.75 – 2.38)	0.95 (0.85 – 1.06)
	Bleeker 2007[97]	All SBI	26.0	0-36	0.74 (0.56 – 0.96)	1.30 (1.07 – 1.60)
Drowsiness	Offringa 1992[91]	Meningitis	7.4	3-72	1.99 (1.29 – 3.08)	0.65 (0.42 – 1.00)
	Oostenbrink 2001[98]	Meningitis	38.7	1-180	2.43 (1.82 – 3.26)	0.37 (0.25 – 0.66)
Cyanosis	Oostenbrink 2001[98]	Meningitis	38.7	1-180	50.2 (2.97 – 846)	0.88 (0.81 – 0.95)
	Nijman 2013[96]	Pneumonia	14.5	1-180	5.24 (3.33 – 8.25)	0.89 (0.84 – 0.94)
	Nijman 2013[96]	Other SBI	14.5	1-180	0	1.03 (1.02 – 1.03)
Poor circulation	Oostenbrink 2001[98]	Meningitis	38.7	1-180	3.71 (2.32 – 5.93)	0.56 (0.44 – 0.73)
	Bleeker 2007[97]	All SBI	26.0	0-36	2.39 (1.50 – 2.83)	0.83 (0.73 – 0.94)
	Nijman 2013[96]	Pneumonia	14.5	1-180	1.14 (0.54 – 2.43)	0.99 (0.96 – 1.03)
	Nijman 2013[96]	Other SBI	14.5	1-180	2.14 (1.22 – 3.75)	0.96 (0.92 – 1.00)
Abnormal chest signs	Mahabee-Gittens 2005[92]	Pneumonia	8.6	2-59	2.21 (0.89 – 5.50)	0.93 (0.84 – 1.04)

Table 3: Performance characteristics of individual clinical signs for the diagnosis of SBI in children presenting to the ED. LR+: Positive likelihood ratio, LR-: Negative likelihood ratio. 'Other SBI' are SBI other than pneumonia. LRs were calculated using the epiR package in R[100]

Indicator	Reference	Outcome diagnosis	Proportion with SBI (%)	Age range/ months	LR+	LR-
Increased work of breathing	Taylor 1995[93]	Pneumonia	7.3	0-24	3.08 (2.41 – 3.94)	0.37 (0.23 – 0.60)
	Mahabee-Gittens 2005[92]	Pneumonia	8.6	2-59	1.11 (0.70 – 1.74)	0.96 (0.78 – 1.18)
	Bleeker 2007[97]	All SBI	26.0	0-36	3.60 (2.06 – 6.28)	0.81 (0.72 – 0.91)
	Nijman 2013[96]	Pneumonia	14.5	1-180	1.95 (1.32 – 2.88)	0.92 (0.87 – 0.98)
	Nijman 2013[96]	Other SBI	14.5	1-180	0.16 (0.04 – 0.63)	1.07 (1.05 – 1.09)
Meningism	Offringa 1992[91]	Meningitis	7.4	3-72	275 (16.7 – 4526)	0.52 (0.35 – 0.76)
	Oostenbrink 2001[98]	Meningitis	27.5	1-180	2.57 (2.16 – 3.06)	0.01 (0 – 0.15)
	Nielsen 2001[94]	MCD	18.8	1-192	13.9 (5.41 – 35.60)	0.61 (0.47 – 0.79)
Petechial rash	Offringa 1992[91]	Meningitis	7.4	3-72	83.7 (4.50 – 1475)	0.86 (0.73 – 1.01)
	Oostenbrink 2001[98]	Meningitis	27.5	1-180	6.18 (2.68 – 14.3)	0.81 (0.73 – 0.91)
	Nielsen 2001[94]	MCD	18.8	1-192	9.00 (5.26 – 15.3)	0.28 (0.16 – 0.48)
	Wells 2001[95]	Bacteraemia	11.0	0-180	7.00 (4.60 – 10.7)	0.19 (0.08 – 0.46)
	Thayyil 2005[101]	All SBI	11.1	1-36	8.90 (2.63 – 30.4)	0.75 (0.63 – 0.91)
Seizures	Joffe 1983[90]	Meningitis	5.4	6-72	5.90 (1.79 – 19.0)	0.80 (0.59 – 1.08)
	Offringa 1992[91]	Meningitis	7.4	3-72	3.50 (1.69 – 7.17)	0.76 (0.58 – 1.00)
	Oostenbrink 2001[98]	Meningitis	27.5	1-180	1.68 (0.66 – 4.27)	0.96 (0.90 – 1.04)
Reduced conscious level	Offringa 1992[91]	Meningitis	7.4	3-72	155 (9.03 – 2677)	0.73 (0.57 – 0.93)

Table 3 (cont): Performance characteristics of individual clinical signs for the diagnosis of SBI in children presenting to the ED. LR+: Positive likelihood ratio, LR-: Negative likelihood ratio. MCD: Meningococcal Disease. LRs were calculated using the epiR package in R[100]

1.6.3. Biomarkers of SBI in children

The limitations of clinical variables in diagnosing SBI in children have encouraged the use of biomarkers to support the diagnostic process. A biomarker is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"[102]. Strictly speaking, the clinical variables described in the previous section could be termed biomarkers, but I will use the term with specific reference to laboratory-measured biomarkers from clinical samples. Biomarkers may be used alone or in combination to allow classification of an individual to a group with defined characteristics.

An improved understanding the complexity of the host response to serious infection has driven the development of biomarkers to provide diagnostic or prognostic information. Almost 200 such biomarkers have now been related to sepsis[103]. I will summarise current understanding of the host response before describing biomarkers of SBI currently used in children, and concluding with discussion of the small number of newer biomarkers of SBI which have been evaluated in the children's ED.

1.6.4. The host response to serious infection

The interaction between host and pathogen results in a complex and dynamic cascade of pro-inflammatory and regulatory anti-inflammatory signals (Figure 2), the outcome of which is one of pathogen eradication, colonisation, or otherwise overwhelming infection and host demise. The likelihood of each of these possible outcomes depends upon both host (genetic or environmental) and pathogen factors (such as virulence). Key mediators of the host response are described below.

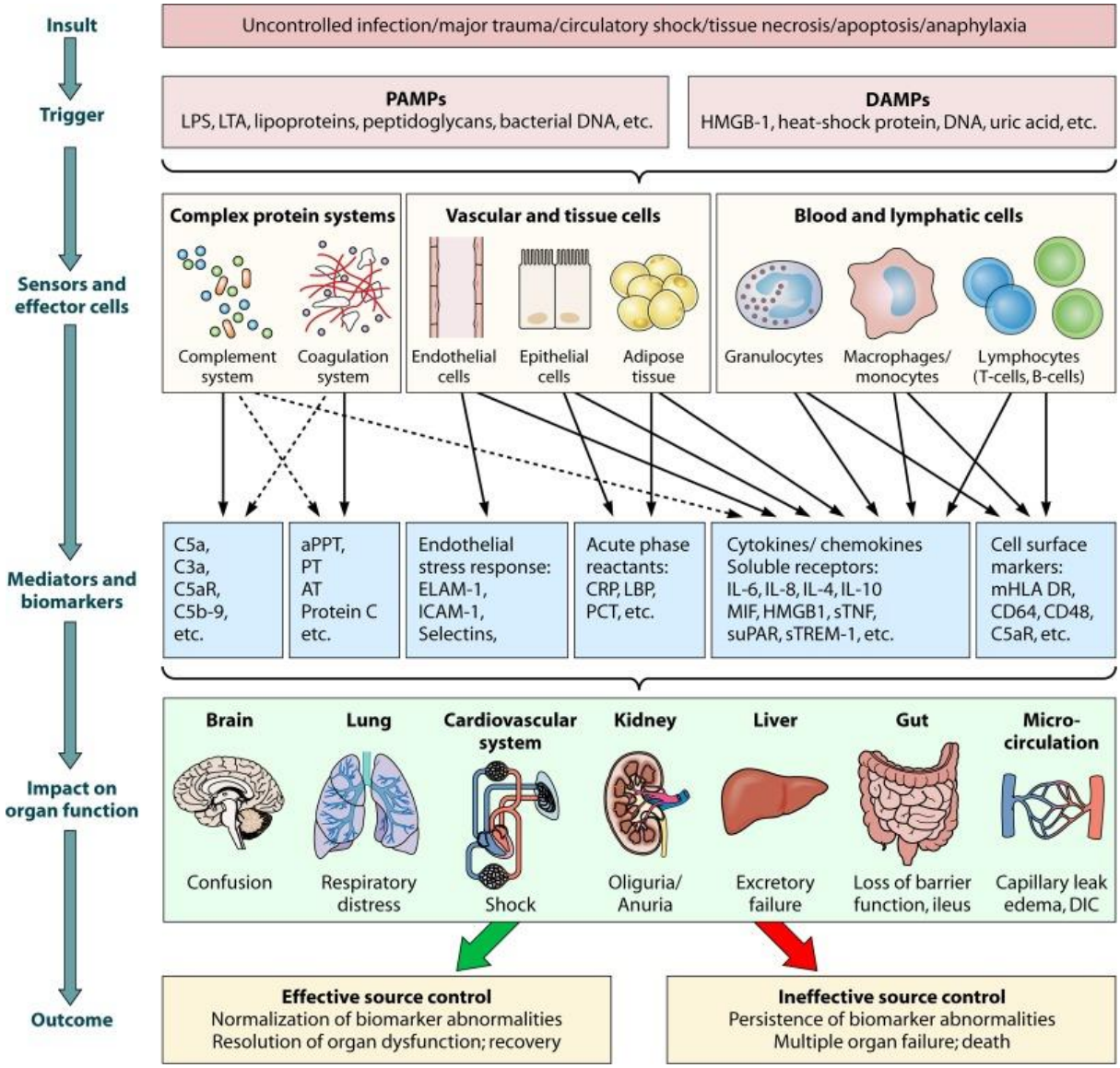


Figure 2: A schematic of the host inflammatory response[104].

1.6.4.1. Toll like receptors

Toll like receptors (TLRs) are one of a family of pattern recognition receptors which act as a first line of the innate immune system. They recognise pathogen-associated molecular patterns, such as lipopolysaccharide (LPS), as well as endogenous peptides released by tissue damage. TLR expression is up-regulated in sepsis[105]. TLRs induce Interleukin-1β (IL-1β), an inflammatory cytokine, and activate "inflammasomes" such as caspases. Caspases regulate apoptosis - that is, programmed cell death - induce other pro-inflammatory cytokines, and also contribute to host defence by up-regulating the production of reactive oxygen species[106]. Various signal transduction enzymes act as downstream regulators of TLR signalling. Depending on target cell type, phosphoinositide 3-kinase (PI3K) can act both positively, to induce pro-inflammatory cytokines such as IL-1β, IL-6, and IL-8 from monocytes, and negatively, inhibiting their production from neutrophils[107]. TLRs also act through Rho GTPases to mediate leucocyte migration, to activate NF-κB and thereby to up-regulate inflammatory cytokine transcription[108]. NF-κB is an important transcription factor which regulates inflammation, cell survival and proliferation[109].

1.6.4.2. Leucocytes

Leucocytes such as neutrophils and macrophages are important cells of the innate system with the capacity to degrade internalised pathogens by generating reactive oxygen species[110], and are recruited to sites of infection. Migration of neutrophils to extravascular sites requires interaction with activated endothelium via adhesion molecules such as beta-2 integrin (CD11/CD18 complexes)[111]. Activation of neutrophils is a necessary and effective mechanism to achieve intracellular killing of pathogens, but neutrophil activation also has the potential for injury to the host. Extracellular neutrophil elastase can impair fibrinolysis and influence the coagulation cascade[112]. Neutrophil-mediated injury is well described, and the inflammatory response is mitigated by neutrophil apoptosis. In sepsis, however, inflammatory cytokines induce prolonged neutrophil survival and activation[113].

Neutrophil Gelatinase-associated Lipocalin (NGAL) is a component of neutrophil granules and has a role in innate immunity through its ability to bind siderophores

required by many bacterial pathogens to scavenge iron from the host [114]. It is highly upregulated by inflammatory stimuli via NF-κB [115] and is considered to have an antibacterial iron depletion role[116] as part of the innate immune response. CD64 is a membrane receptor expressed on macrophages and monocytes. In sepsis, it is highly upregulated on neutrophils[117].

1.6.4.3. Inflammatory cytokines

Inflammatory cytokines such as IL-1β, Tumour Necrosis Factor-α (TNF-α), IL-6, IL-8 may be released directly from leucocytes, or via the above 'inflammasome' or TLR signalling pathways. Their effects are to induce further leucocyte migration and activation, and to activate endothelial surfaces by up-regulating the expression of adhesion molecules and increasing vascular permeability[118,119].

Cytokines with a particular role in stimulating leucocyte migration are termed chemokines[120]. IL-8 (termed CXCL8 according to chemokine nomenclature) is a potent chemoattractant of neutrophils, causing neutrophil migration to sites of infection, and inducing phagocytosis[121]. Other significant chemotactic proteins include Monocyte Chemoattractant Protein-1 (MCP-1, or CCL2), Macrophage Inflammatory Protein 1-β (MIP-1β, or CCL4) and RANTES (CCL5), all of which act primarily on monocytes and macrophages[122], but also recruit T-lymphocytes[123].

High Mobility Group Box-1 (HMGB-1) is a nuclear protein which has a role in maintaining chromosomal DNA stability[124]. It also contributes to angiogenesis and tissue repair[125]. In response to inflammatory stimuli (such as IFN-γ), HMGB-1 is released by macrophages and monocytes. This release occurs late in the inflammatory response, increasing to a steady state at 16-32h and persisting in adults with septic shock[126]. Another important nuclear protein in the regulation of sepsis is Peroxisome Proliferator-activated Receptor-γ (PPARγ). In mouse models, PPARγ modulates inflammation by preventing the release of inflammatory cytokines IL-1β, IL-6 and TNF-α via the TLR signalling pathway[127]. Furthermore, the use of PPARγ agonists reduces levels of HMBG-1 and improves survival in mice with septic shock[128,129]. Both HMGB-1 and PPARγ are involved in the subsequent expression of Resistin. Resistin is an adipokine first related to insulin resistance in

mice[130]. In humans, Resistin expression occurs predominantly in macrophages, monocytes and neutrophils[131,132]. Circulating Resistin levels peak 8-16h after the administration of LPS in healthy subjects, mediated by pro-inflammatory cytokines and NF-κB [133]. HMGB-1 is a potent inducer of Resistin in monocytes, and mediates the persistence of elevated Resistin levels in patients with sepsis on ICU[134], while PPARγ activity is increased and correlates with Resistin levels in children with sepsis[135].

1.6.4.4. Anti-inflammatory cytokines

Anti-inflammatory cytokines are important mediators of the inflammatory response. IL-10 is released by leucocytes, and suppresses the production of the pro-inflammatory cytokines IL-1β, TNF-α, IL-6 and IFN-γ[136]. Further, IL-10 stimulates production of IL-1 receptor antagonist (IL-1Ra) and soluble TNF receptors (sTNFRs) which act to neutralise their pro-inflammatory effects[137]. Transforming Growth Factor –β (TGF-β) has also been shown to attenuate the inflammatory response by suppressing the release of pro-inflammatory cytokines, and by stimulating the production of IL-1Ra and sTNFRs[138].

The observation that the early administration of IL-10 and TGF-β improved survival in murine models of sepsis illustrated the importance of anti-inflammatory cytokines in the host response to severe infection. Consistent with this, immunoneutralisation of IL-10 led to elevated IL-6 and TNF-α, and was associated with worse outcomes[139]. Interestingly, however, late inhibition of IL-10 in this model improved survival[140], suggesting that the balance between pro- and anti-inflammatory cytokines is dynamic. In adults presenting to the ED with acute febrile illnesses an anti-inflammatory cytokine profile (characterised by a high IL-10 to TNF-α ratio) is associated with mortality[141]. In children with MCD, an anti-inflammatory profile (high IL-1Ra to TNF and IL-6 ratios) is associated with more severe disease[142]. It is hypothesised that following the initial hyperinflammatory state of early sepsis, patients enter a more protracted state of immunoparesis and immunosuppression which is an important contributor to late mortality[143].

1.6.4.5. Acute phase reactants

Acute phase reactants are typically induced by inflammatory cytokines. C-reactive Protein (CRP) is the best known acute phase reactant and is in widespread clinical use as a diagnostic test. The name was derived from its discovery, bound to the C polysaccharide of the pneumococcal cell wall in adults with pneumonia[144]. It is a 'pentraxin', a secreted pattern recognition receptor synthesised in the liver following induction by pro-inflammatory cytokines, particularly IL-6[145,146]. It binds microbial peptides and damaged cells and mediates their destruction through its interaction with the core complement component C1q[145]. Levels of CRP peak at 36-50h in response to an inflammatory signal[147].

Procalcitonin (PCT) is a 116 amino acid protein derived most commonly from neuroendocrine cells including the C-cell of the thyroid with which it was first associated[148]. Whilst Calcitonin is thought to have a role in calcium metabolism, and bone resorption through its action on the osteoclast[149], the action of PCT remains unclear. In normal conditions the Calcitonin gene CALC-I, located on chromosome 11, is selectively expressed in neuroendocrine tissues, and transcribes the prohormone PCT. In these circumstances, Calcitonin is then produced by the action of proteolytic enzymes on PCT. PCT and its constituent proteins are present in low concentrations in normal subjects, and become markedly elevated in a number of conditions (see Table 4). Under such conditions, including sepsis, CALC-I is expressed systemically and Calcitonin mRNA detected in a wide of variety of tissues[150]. The resultant increase in the level of PCT occurs in the absence of the enzymatic pathways which control Calcitonin and its precursors in neuroendocrine tissue, and is not mirrored by a corresponding increase in the Calcitonin level. Its role in sepsis is unclear though it has been a target of immunomodulation in animal models[151]

Infection	Bacterial Parasitic Fungal Viral
Neoplasm	Neuroendocrine tumours Medullary Thyroid Carcinoma Small Cell Lung Cancer Carcinoid tumour
Inflammation	Pancreatitis Burns Heat Stroke
Trauma	
Surgery	

Table 4: Conditions which elevate PCT[152].

Ferritin is an iron storage protein whose synthesis is increased by the pro-inflammatory cytokines IL-6 and IL-8. It is elevated in systemic inflammation, and is associated with mortality in children with severe sepsis[153]. Mannose Binding Lectin (MBL) is synthesised primarily by the liver, and binds carbohydrates on the surface of pathogens before activating complement via MBL-associated serine proteases[154]. This "pattern recognition" role is an important feature of innate immunity. Mutations in the MBL2 gene have been associated with frequent and recurrent infections[155], and low levels of MBL in paediatric sepsis have been associated with severity of illness[156].

Serum amyloid A is another acute phase protein produced in the liver in response to inflammatory stimuli. It has multiple functions relating to inflammatory modulation, and cholesterol metabolism[157]. Haptoglobin scavenges cell free haemoglobin and heme, which are increased in sepsis and mediate tissue damage[158]. In animal models this scavenging attenuates the inflammatory response and decreases mortality[159]. Haemoglobin-haptoglobin complexes are endocytosed

by macrophages via the haemoglobin scavenger receptor CD163[160]. The soluble form of the receptor (sCD163) is shed from the cell membrane in response to inflammatory stimuli[161], and levels have been found to predict prognosis in pneumococcal bacteraemia[162].

1.6.4.6. Complement

Complement is a sophisticated and highly regulated network of enzyme pathways. In the classical pathway, presentation of microbial peptides to C1q initiates a cascade which results in the production of C3a and C5a[145]. The effects of this complement activation are multitude, including chemotaxis of leucocytes, endothelial activation, and induction of phagocytosis through opsonisation of microbial peptides and apoptotic cells. Complement interacts with other components of the innate immune system, including TLRs and the coagulation cascade, as well as priming the adaptive immune system, in particular the humoral response[145].

1.6.4.7. Coagulation and the endothelium

The inflammatory milieu in sepsis is prothrombotic[163]. The endothelium, which in normal conditions acts to prevent thrombus formation, is activated by inflammatory cytokines, LPS and complement[164]. Activated endothelium stimulates platelet adhesion and platelet aggregation, and releases von Willebrand factor (vWF) into the circulation[165]. Activated endothelial cells are more likely to undergo apoptosis, and along with vWF, release angiopoietins into the circulation. Angiopoietins are endothelial growth factors. Ang-1 has a role in enhancing endothelial survival and barrier integrity. Ang-2, by contrast, propagates inflammatory pathways by sensitising endothelial cells to TNF-α, including an up-regulation of adhesion molecules[166]. The ratio of Ang-1 to Ang-2 has been suggested as a prognostic marker of serious infection[167,168].

Other inflammatory mechanisms tip the balance of coagulation in favour of thrombosis. Complement induces the expression of tissue factor on endothelial cells, and inhibits fibrinolysis[169]. Neutrophils prevent inhibitors of the tissue factor pathway, localising coagulation to small vessels, thereby preventing pathogen

dissemination[170]. Various mechanisms inhibit anticoagulant pathways. In particular the protein C pathway, which limits coagulation by the inactivation of clotting factors Va and VIIIa, is impaired. Production of protein C is reduced, consumption increased, and activation limited by a reduction in the expression of thrombomodulin on endothelial surfaces mediated by TNF-α[171].

In return, coagulation pathways influence inflammation through protease-activated receptors (PARs). Thrombin induces IL-6 from endothelium via PAR-1 and PAR-2[172]. Activated Factor VII can also induce IL-6 and IL-8 production[173]. Clotting factors cleave C1 and activate complement[174], while thrombin activates C5[175]. In response activated protein C down-regulates pro-inflammatory signals via PAR-1[176], while promoting endothelial integrity[177].

Adhesion molecules play a critical role in leucocyte migration to sites of infection, and across the vascular endothelium. Those most closely associated with the host response to sepsis are the selectins (E-selectin, L-selectin and P-selectin) along with intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1). With the exception of L-selectin (which is expressed on leucocytes) these molecules are expressed on the endothelium and mediate endothelial adhesion, leucocyte rolling, and migration across endothelial surfaces. All are up-regulated by inflammatory stimuli TNF-α, IL-1β, IL-6 and LPS[178]. Upon endothelial activation, these adhesion molecules are promptly shed from the surface, and soluble isoforms become detectable in the plasma. It is proposed that this shedding limits leucocyte-endothelial interaction, exerting a protective effect by mitigating the inflammatory cascade locally[179]. Elevated levels of E-selectin and L-selectin have been associated with improved outcomes in sepsis in children[180] and adults[181]. A similar pattern of shedding in sepsis has been observed with ICAM-1 and VCAM-1. In both cases, shedding is postulated to be a protective mechanism limiting local inflammation[182]. Whilst the highest levels of ICAM-1 are associated with improved outcome in septic children[180], adults with the most elevated levels of VCAM-1 had the highest mortality from sepsis[183]. To date the relationship between soluble adhesion molecules and diagnosis and prognosis in sepsis remains unclear.

1.6.4.8. Summary of the host response

The complexity and heterogeneity of the host response to serious infection underlie the many and varied presentations of children to the ED. It is this heterogeneity that makes the identification of reliable diagnostic tools so challenging. Of the many mediators of the host response described above, few have been demonstrated to be sufficiently specific to infection to provide adequate diagnostic accuracy.

In the subsequent section, I will discuss the current use of biomarkers of SBI in the children's ED, before summarising the evidence for newer potential markers in this context.

1.6.5. Biomarkers of childhood SBI in current clinical practice

Few biomarkers are in routine clinical use for the diagnosis of SBI in children. In its "Feverish illness in children" guideline, NICE recommends the use of CRP and WCC in the evaluation of febrile children under the age of 5 years. The most recently updated guideline includes a meta-analysis suggesting that PCT may perform moderately better than CRP in the diagnosis of childhood SBI, but that the availability and cost of the test currently preclude its widespread implementation[184].

Table 5 summarises the performance characteristics of prospective studies of CRP, WCC and PCT for the diagnosis of SBI in children in the ED. For an explanation of terms used to quantify diagnostic accuracy, including the likelihood ratios used in Table 5, see Section 2.6.1.Few studies included children older than 3 years. A number specifically considered young infants less than 3 months of age. Most of the biomarker evaluations were performed in populations of children with a substantial risk of SBI (only one had a rate of SBI of less than 10% of participants). Together these characteristics limit the applicability of the findings in all children presenting to the ED with acute febrile illness.

1.6.5.1. C-reactive Protein

CRP is commonly used in clinical practice in the UK, as recommended by the NICE "Feverish Illness in Children" guidelines. As such, it has been extensively investigated as a marker of SBI in children in the ED. Quantitative point of care tests, such as the Afinion CRP (Alere, Waltham, USA) are now commercially available, increasing access to the use of the test. Despite this widespread use, the value of CRP in discriminating between SBI and self-limiting infections is limited when used in isolation. Meta-analysis included in the NICE guideline estimated a pooled positive likelihood ratio (PLR) of 3.1 (95% CI 2.5 to 3.7) and negative likelihood ratio (NLR) of 0.27 (95% CI 0.18 to 0.42) for the diagnosis of SBI in children[184]. These estimates are comparable to those reported by van den Bruel *et al*[185].

1.6.5.2. White cell count

White cells (or leucocytes) are a central component of the host response. Abnormalities of the WCC are associated with a range of immune, inflammatory and haematological conditions and so its measurement as part of the full blood count is a common part of the evaluation of the unwell child. For the purposes of diagnosing SBI, however, white cell count is insufficiently discriminatory. The largest single study of its diagnostic accuracy in children under the age of 5 estimated a PLR of 1.93 (95% CI 1.75 to 2.13) and NLR of 0.70 (95% CI 0.65 to 0.75) at a WCC threshold of 15 x10^9/l, consistent with NICE guidance[186]. Despite its limitations, it has been incorporated into clinical rules aimed at identifying young infants at risk of serious infection, such as the Rochester criteria (Section 1.6.8)[187].

The Neutrophil count is a subset of the WCC. As an important cell of the innate immune response, abnormalities of the Neutrophil count have been evaluated as a marker of SBI in children. Perhaps unsurprisingly, its diagnostic accuracy appears comparable to that of WCC[186].

1.6.5.3. Procalcitonin

PCT has been extensively evaluated in children since its first description in hospitalised children with suspected sepsis[188]. Its rapid increase in response to serious infection, and resolution with appropriate therapy suggest a valuable diagnostic test. PCT performs moderately better than CRP in studies of children presenting to the ED[101,189-192]. In particular it may have a role in children presenting with a short history (<12h) of fever, when CRP is unreliable[190,191]. Like CRP, it remains insufficiently discriminatory to use in isolation. A pooled PLR of 4.1 (95% CI 2.9 to 5.7), and NLR of 0.25 (95% CI 0.16 to 0.38) were estimated by the NICE meta-analysis[184]. Near patient testing is available in the semi-quantitative PCT-Q assay and more recently the quantitative PCT Direct (both Thermo Fisher Scientific, Waltham, USA).

Biomarker	Reference	Proportion with SBI (%)	Threshold	Age range/ months	LR+	LR-
CRP	Berger 1996[193]	23.9	20	<12	2.53 (1.82 – 3.50)	0.25 (0.11 – 0.56)
	Pulliam 2001[194]	18.2	70	1-36	8.30 (3.8 – 27.3)	0.24 (0.09 – 0.65)
	Lacour 2001[189]	22.6	40	<36	3.57 (2.47 – 5.17)	0.14 (0.05 – 0.42)
	Isaacman 2002[195]	11.3	44	3-36	3.28 (2.21 – 4.85)	0.47 (0.29 – 0.75)
	Galetto-Lacour 2003[196]	29.2	40	<36	3.70 (2.28 – 6.01)	0.26 (0.13 – 0.54)
	Fernandez-Lopez 2003[190]	42.6	27.5	1-36	3.15 (2.44 – 4.07)	0.29 (0.21 – 0.40)
	Thayyil 2005[101]	11.1	50	1-36	2.40 (1.40 – 4.12)	0.36 (0.11 – 1.22)
	Hsiao 2006[197]	10.3	9.8	2-6	2.61 (1.81 – 3.76)	0.61 (0.44 – 0.83)
	Andreola 2007[191]	23.0	40	<36	3.79 (2.92 – 4.94)	0.35 (0.26 – 0.49)
	Galetto Lacour 2008[7]	26.7	40	<36	3.35 (2.45 – 4.57)	0.25 (0.14 – 0.43)
	Olaciregui 2009[198]	23.6	30	<3	5.35 (3.63 – 7.89)	0.47 (0.36 – 0.60)
	Manzano 2011[199]	16.4	17.1	1-36	3.01 (2.50 – 3.63)	0.08 (0.03 – 0.24)
Procalcitonin	Lacour 2001[189]	22.6	0.9	<36	4.24 (2.87 – 6.28)	0.09 (0.02 – 0.35)
	Galetto-Lacour 2003[196]	29.2	0.5	<36	3.62 (2.40 – 5.46)	0.09 (0.02 – 0.36)
	Fernandez-Lopez 2003[190]	42.6	0.59	1-36	14.19 (8.37 – 24.1)	0.09 (0.06 – 0.16)
	Thayyil 2005[101]	11.1	0.5	1-36	1.75 (1.22 – 2.50)	0.25 (0.04 – 1.59)
	Andreola 2007[191]	23.0	0.5	<36	3.11 (2.47 – 3.93)	0.35 (0.25 – 0.49)
	Galetto Lacour 2008[7]	26.7	0.5	<36	2.96 (2.33 – 3.80)	0.08 (0.03 – 0.25)
	Maniaci 2008[192]	17.9	0.12	<3	1.28 (1.15 – 1.42)	0.19 (0.05 – 0.74)
	Olaciregui 2009[198]	23.6	0.5	<3	4.94 (3.47 – 7.04)	0.42 (0.31 – 0.56)
	Manzano 2011[199]	16.4	0.2	1-36	2.81 (2.28 – 3.47)	0.21 (0.11 – 0.40)
	Woelker 2012[200]	8.4	0.26	<2	2.57 (1.96 – 3.37)	0.12 (0.02 – 0.79)

Table 5: Performance characteristics of biomarkers for the diagnosis of SBI in children in the ED. LRs were calculated using the epiR package in R[100]

Biomarker	Reference	Proportion with SBI (%)	Threshold	Age range/ months	LR+	LR-
White cell count	Berger 1996[193]	23.9	15	<12	1.67 (1.08 – 2.60)	0.70 (0.48 – 1.02)
	Pulliam 2001[194]	18.2	15	1-36	1.93 (1.14 – 3.26)	0.54 (0.26 – 1.11)
	Lacour 2001[189]	22.6	15	<36	2.96 (1.89 – 4.63)	0.42 (0.24 – 0.72)
	Nademi 2001[201]	29.1	15	0-192	1.95 (0.55 – 6.90)	0.95 (0.85 – 1.06)
	Fernandez-Lopez 2003[190]	42.6	17.1	1-36	2.27 (1.70 – 3.03)	0.60 (0.50 – 0.73)
	Thayyil 2005[101]	11.1	15	1-36	1.07 (0.51 – 2.24)	0.94 (0.45 – 1.95)
	Trautner 2006[202]	19.4	15	0-204	0.87 (0.51 – 1.47)	1.14 (0.72 – 1.80)
	Hsiao 2006[197]	10.3	15.7	2-6	2.43 (1.73 – 3.43)	0.61 (0.44 – 0.83)
	Andreola 2007[191]	23.0	15	<36	2.08 (1.58 – 2.75)	0.65 (0.52 – 0.80)
	Galetto Lacour 2008[7]	26.7	15	<36	1.92 (0.55 – 6.90)	0.66 (0.49 – 0.89)
	Olaciregui 2009[198]	23.6	15	<3	2.39 (1.61 – 3.53)	0.74 (0.62 – 0.88)
	Manzano 2011[199]	16.4	14.1	1-36	2.79 (2.23 – 3.49)	0.26 (0.15 – 0.46)
	De 2013[186]	18.3	15	0-60	1.93 (1.75 – 2.13)	0.70 (0.65 – 0.75)
Neutrophil count	Pulliam 2001[194]	18.2	10.2	1-36	3.00 (1.73 – 5.21)	0.38 (0.16 – 0.87)
	Fernandez-Lopez 2003[190]	42.6	9.9	1-36	2.66 (1.96 – 3.61)	0.56 (0.47 – 0.68)
	Trautner 2006[202]	19.4	10	0-204	1.06 (0.68 – 1.66)	0.93 (0.55 – 1.59)
	Andreola 2007[191]	23.0	10	<36	1.38 (0.95 – 2.00)	0.90 (0.62 – 1.00)
	Manzano 2011[199]	16.4	5.2	1-36	2.17 (1.82 – 2.59)	0.22 (0.11 – 0.44)
	De 2013[186]	18.3	10	0-60	1.87 (1.68 – 2.09)	0.75 (0.71 – 0.80)

Table 5 (cont): Performance characteristics of biomarkers for the diagnosis of SBI in children in the ED. .LRs were calculated using the epiR package in R[100]

1.6.6. Alternative markers of SBI in the children's ED

Despite significant interest in the use of biomarkers to predict serious infection, relatively few have been investigated in children in the Emergency Department. Here, I will summarise the evidence for alternative markers of SBI in the children's ED.

1.6.6.1. Interleukins (IL-1, IL-6, IL-8)

The inflammatory cytokines IL-1, IL-6, and IL-8 have long been considered potential biomarkers of serious infection in view of their central role in the host inflammatory response. Studies of adults with sepsis on ICU which focussed on the prognostic role of the inflammatory cytokines reached varying conclusions. Whilst the cytokines were found be elevated in non-survivors of sepsis, the prognostic value was not greater than that achieved by the use of clinical variables alone[203-205]. Numerous studies have evaluated cytokines in the diagnosis of neonatal sepsis. Following the observation that IL-1Ra (a competitive antagonist of IL-1 at the IL-1 receptor) is present systemically at much higher concentrations than IL-1β[206], most have evaluated IL-1Ra instead of IL-1. Though an early study suggested superior diagnostic accuracy of IL-1Ra and IL-6 to CRP[207], no subsequent studies have validated this finding[208].

In the children's ED, a limited evidence base describes these inflammatory cytokines in the diagnosis of SBI. A small case-control study compared IL-6 to CRP and PCT for the purpose of differentiating viral from bacterial infections, and concluded that IL-6 was inferior to PCT[209]. A study of 124 children under 3 years evaluated IL-1Ra, IL-6 and IL-8 alongside PCT, CRP and a clinical score. It concluded that IL-1Ra and IL-8 failed to differentiate SBI from self-limiting infection, and that IL-6 was inferior to PCT and CRP[189]. A subsequent study by the same authors corroborated these findings[196].

1.6.6.2. Neutrophil Gelatinase-associated Lipocalin

NGAL was first identified as a marker of acute kidney injury in patients on the intensive care unit[210]. It was subsequently found to be more significantly elevated in sepsis than in other inflammatory states[211-213]. In adults presenting to the ED with

suspected sepsis, an optimal biomarker panel included NGAL, protein C, and IL-1Ra. The area under the curve for this 'sepsis score' was 0.80 for severe sepsis, 0.77 for septic shock, and 0.79 for death[214] Using transcriptomic analysis in Malawian children presenting with signs of pneumonia and meningitis, NGAL, along with PCT and Resistin were found to be differentially expressed in children with SBI and measurement of the protein products discriminated between those with SBI and those without. Combination of the three markers achieved an AUC of 0.90 for the diagnosis of SBI[215]. In Kenyan children, the combination of NGAL with haptoglobin achieved an AUC of 0.82 for the diagnosis of bacterial pneumonia[216].

1.6.6.3. Resistin

Resistin is significantly elevated in intensive care patients with sepsis[217]. Two small case-control studies in the neonatal unit demonstrated differences between neonates with proven sepsis and healthy neonates, though to date no high quality diagnostic accuracy studies have been done in this setting[218,219]. A single study of 37 adults with sepsis in ED identified that persistent elevation of Resistin, NGAL and IL-8 beyond 24h after presentation predicted the presence of severe sepsis and septic shock[220]. The only published study to describe the performance of Resistin in children in the ED is the above study of Malawian children presenting with signs of pneumonia and meningitis, in which Resistin, together with PCT and NGAL predicted the presence of SBI[221].

1.6.6.4. Neutrophil CD64

CD64 expression on neutrophils is increased in neonates with culture proven sepsis compared with culture negative neonates who undergo a sepsis evaluation[222]. A small study of 82 critically unwell children and neonates concluded that CD64 expression was superior to CRP and PCT in the diagnosis of 'sepsis'[223]. A further study of 38 pre-term infants quantified the 'proportionate reduction in uncertainty' of the presence of infection and suggested that CD64 expression performed better than CRP or PCT in the diagnosis of infection[224]. Only one study of 70 febrile children (under the age of 3 years) has evaluated CD64 expression in the ED, reporting a sensitivity of 95% for SBI, with a specificity of 47%[225].

1.6.6.5. Soluble urokinase plasminogen activator receptor (suPAR)

Soluble urokinase plasminogen activator receptor (suPAR) is the cleaved form of the membrane bound urokinase plasminogen activator receptor, expressed on neutrophils, and macrophages[226]. suPAR has to date demonstrated only limited value in the identification of sepsis in critically ill adults[227,228]. Two small studies of suPAR for the prediction of SBI in NICU revealed modest performance[229,230]. A single study evaluated the use of suPAR in the prediction of severity of pneumonia in children, though did not report diagnostic accuracy[231], whilst another evaluated the prognostic value of suPAR to predict upper urinary tract involvement in children with UTI[232]. It concluded that the use of suPAR could predict those children who may have upper urinary tract involvement and require further investigation with DMSA following a UTI.

1.6.7. The combination of variables in clinical prediction

Considerable diagnostic uncertainty remains when individual clinical signs or biomarkers are used to diagnose SBI in the children's ED. The combination of clinical and biomarker variables in the form of a clinical prediction rule (CPR, or clinical prediction model) may enhance the ability of clinicians to identify serious infection. Clinical risk prediction such as this has the potential to provide valuable diagnostic information, but requires a robust approach to development and validation[233]. The diagnosis of serious infection in febrile children has been a common question addressed by CPRs in children, but very few have undergone external validation and impact analysis[234].

I will summarise the evidence supporting the use of CPRs in the diagnosis of SBI in the children's ED.

1.6.8. Clinical prediction rules for SBI in the children's ED

An early CPR, widely adopted as the "Yale Observation Scale" (YOS) was developed in young children and combined six characteristic of children with fever[235]. The

score evaluated the “quality of cry”, “reaction to parental stimulation”, “state variation”, “colour”, “hydration”, and “social response” on categorical scales. The study recruited 312 children under the age of 2 years, of whom 37 had SBI. Using a threshold of 10 points, the score demonstrated a positive likelihood ratio of 3.8 (95% CI 2.75 – 5.37), and an encouraging negative likelihood ratio of 0.15 (95% CI 0.03 – 0.44). External validation studies however, mostly in children up to 3 years of age, failed to reproduce these results[101,189,191,196,197,236-238].

A number of early studies examined the value of CPRs in ruling out SBI in young infants. These infants, less than 3 months of age, were considered at high risk of SBI and had conventionally been admitted to hospital and treated with empirical antibiotics. The first of these, which established the ‘Rochester’ criteria, determined that in previously healthy young infants a ‘normal’ clinical examination alongside a normal white cell count, and urinalysis could be used to identify ‘low-risk’ infants suitable for observation. The low-risk criteria performed well in this regard. Only 1/144 infants considered at low risk was found to have a serious infection, and no adverse outcomes were reported[187]. These findings were subsequently validated in a different population[239].

Validation studies of the Rochester criteria and modifications of these (which incorporated the use of CRP or Erythrocyte Sedimentation Rate), similarly suggested the safety of this approach[239-245]. An alternative approach to young infants over one month of age utilized the YOS first evaluated in older children[235]. In this age group, the YOS alone failed to be sufficiently sensitive to rule out SBI[236]. A later study however combined the YOS with WBC, urinalysis and CSF microscopy to yield a low-risk group (the Philadelphia protocol)[237], and in 3 studies which used this approach, the criteria safely ruled out SBI (negative LR <0.2)[237,244,246]. Only 2/422 (0.5%) of low-risk infants were misclassified, and none had adverse outcomes. A further variation of this approach - the Milwaukee protocol - failed to demonstrate the safety of their approach (negative LR 0.15, 95% CI 0.02 - 1.02)[247].

A large study of more than 5000 young infants derived a novel prediction model combining clinical variables along with urinalysis, white blood cell count, and CSF microscopy. The sensitivity of the model for SBI was 82%. Nearly one fifth of SBI was misclassified as low-risk, including 14/59 infants with bacteraemia, and a further infant with meningitis. No external validation has since been performed[248]. Details of the low-risk criteria applied are detailed in Table 6, and the performance characteristics of the use of low-risk criteria for SBI in young infants are summarized in Table 7.

Low-risk protocol	Age group / days	Criteria
Rochester[187]	0-90	Normal clinical exam 5 < WCC < 15 x10^9/litre Urine microscopy <10 WCCs per high power field
Philadelphia[237]	28-56	YOS ≤10 WCC <15 x10^9/litre Urine microscopy <10 WCCs per high power field CSF <8 WCCs per high power field CXR no infiltrate
Milwaukee[247]	28-56	Normal clinical exam CSF<10 WCCs per high power field Negative CSF Gram stain WCC <15 x10^9/litre Urine microscopy <10 WCCs per high power field Negative urine Gram stain
Decision tree[248]	0-90	Urinalysis (LE or nitrite) negative Urine microscopy <5 WCCs per high power field 4.1<WCC <20 x10^9/litre Temp <39.6°C Age >13 days

Table 6: Low-risk criteria applied to young infants in order to rule out SBI. WCC: Peripheral blood White cell count. YOS: Infant observation Score. CSF: Cerebrospinal fluid. CXR: Chest Xray. LE: Leucocyte esterase

Few CPRs for the diagnosis of SBI in older children feature commonly in clinical use. In the UK, the management of children with fever is based upon expert guidance - the NICE "Feverish illness in children" guideline, designed for use in children under the age of 5[184]. The guideline identifies clinical signs which may predict the presence of serious infection, and allocates each to a "traffic light" based risk category. Recently, the value of the NICE guideline has been evaluated in two large prospective studies in the children's ED[249,250]. In each, the absence of any 'amber' or 'red' signs had some value in ruling out serious infection, though the presence of the same features had little role in ruling in serious infection.

The Manchester triage system (MTS) is a well-established triage tool, though not designed to detect SBI in children. In common with the NICE guidelines, the MTS demonstrates limited ability to discriminate between SBI and self-limiting infection. In one study, the two most urgent categories appeared to have some value for the ruling in of serious infection (where the outcome was 'serious or intermediate' infection)[249], though was of limited value for the diagnosis of SBI in a second study with a lower proportion of serious outcomes[251].

Recent studies have sought to derive new risk prediction models combining clinical variables and laboratory tests[7,9,96]. The performance characteristics of these are summarised in Table 8. Though encouraging diagnostic performance has been demonstrated in the populations in which the models have been derived, few have performed as well in external validation studies[252].

A large UK based study[9] derived (n=1664) and internally validated (n=287) a prediction rule based on clinical variables. Using a low-risk threshold the model demonstrated a negative likelihood ratio of 0.4 (95% CI 0.3 – 0.6), and a negative predictive value of less than 2%. The analysis was undertaken on a historic dataset, prior to the introduction of the pneumococcal conjugate vaccine, and with a low prevalence of SBI.

A small study[7] in children less than 3 years derived (n=135) and internally validated (n=67) a prediction rule ("Lab score") combining C-reactive protein (CRP), Procalcitonin (PCT) and urine dipstick. In this study the likelihood of SBI was approximately 25%. At a low-risk threshold, the negative likelihood ratio of 0.07 (95% CI 0.02 – 0.21), is promising. The authors suggested that use of the Lab score would have reduced antibiotics by an estimated one third. External validation was undertaken in a historical dataset from another European country, demonstrating similar performance characteristics[253]. In a further validation study of more than 1000 young infants less than 3 months of age, the Lab score demonstrated value in ruling in SBI, but limited ability to rule out SBI[254].

The largest study to derive a clinical prediction model for SBI in children included more than 15000 children under the age of 5 years[6]. The study used multinomial regression to develop risk prediction models for each diagnosis of UTI, pneumonia, and bacteraemia. The models were derived before internal validation in more than 5000 children. The models demonstrated reasonable discrimination in the validation samples, with areas under the ROC curve of 0.78, 0.84, and 0.74 for the diagnosis of UTI, pneumonia and bacteraemia respectively. The authors proposed that the identification of appropriate rule out thresholds as part of a computer based decision tool could be used to rationalise decision-making, though to date no evaluation of the impact of the model upon clinical decision-making has been published.

A large study across three populations in Europe used a similar model building approach, and included an external validation population[96]. The authors derived models for the prediction of pneumonia, and 'other SBIs'. The overall performance of the models showed good discrimination with an AUC 0.81 and 0.86 for the prediction of pneumonia and 'other SBIs' respectively, and good calibration. Discrimination of 'other SBIs' was less good in the external validation population (AUC 0.69). In the validation group, it proved possible to identify low-risk thresholds

below which it was possible to rule out pneumonia (negative LR of 0.12) though a low-risk threshold of <2.5% for other SBIs only achieved a negative LR of 0.59. The study demonstrated the additive value of CRP on a model based upon clinical variables alone.

The above published CPRs for the diagnosis of SBI were recently evaluated in a validation study[252]. In this, the authors applied CPRs derived in one published study, to other prospectively recruited cohorts. In general, the performance of the published rules was less good in the validation samples. In particular, the YOS failed to demonstrate adequate sensitivity, while the NICE guideline appeared to be sensitive, but at a cost of identifying many false positives (non-specific). The authors concluded that the use of such guidelines as NICE in low and intermediate risk settings (including primary care) may allow clinicians to rule out serious infection, but that their value in the higher risk setting of the ED was less clear[252].

Score	Reference	Number of subjects	Proportion with SBI (%)	Age range/ days	LR+	LR-
Yale IOS	Baker 1990[236]	126	29.4	28-56	2.27 (1.32 – 3.90)	0.68 (0.49 – 0.93)
Rochester	Dagan 1985[187]	233	9.9	0-90	3.00 (2.42 – 3.72)	0.06 (0.01 – 0.44)
	Dagan 1988[239]	237	8.9	0-60	3.21 (2.63 – 3.91)	0
	Jaskiewicz 1994[240]	802	7.1	0-60	1.85 (1.68 – 2.03)	0.15 (0.07 – 0.35)
	Chiu 1994[241]	254	17.7	0-31	2.07 (1.67 – 2.57)	0.29 (0.16 – 0.56)
	Chiu 1997[242]	119	16.4	4-28	2.58 (2.15 – 3.09)	0.04 (0.01 – 0.27)
	Byington 2004[243]	1378	9.4	0-90	1.41 (1.32 – 1.51)	0.26 (0.15 – 0.45)
	Garra 2005[244]	259	25.9	0-56	1.54 (1.37 – 1.73)	0.08 (0.02 – 0.32)
	Marom 2007[245]	386	24.0	0-28	2.44 (2.11 – 2.81)	0.02 (0 – 0.11)
Philadelphia	Baker 1993[237]	747	8.7	28-56	1.70 (1.58 – 1.82)	0.04 (0.01 – 0.26)
	Baker 1999[246]	422	10.2	28-56	1.36 (1.28 – 1.45)	0
	Garra 2005[244]	181	21.5	28-56	1.27 (1.14 – 1.41)	0.11 (0.02 – 0.78)
Milwaukee	Bonadio 1993[247]	534	4.5	28-56	1.33 (1.20 – 1.47)	0.15 (0.02 – 1.02)
Decision tree	Bachur 2001[248]	5279	7.1	0-90	3.70 (3.45 – 3.97)	0.23 (0.18 – 0.28)
'Lab score'	Bressan 2012[254]	1012	28.3	0-90	10.5 (7.46 – 14.7)	0.51 (0.45 – 0.57)

Table 7: Clinical prediction rules to identify young infants at low risk of SBI. LRs were calculated using the epiR package in R[100]

Score	Reference	Outcome diagnosis	Proportion with outcome (%)	Age range/ months	LR+	LR-
NICE traffic light	Thompson 2009[249]	'Serious infection'	44.7	3-192	1.20 (1.10 – 1.30)	0.50 (0.40 – 0.70)
	De 2013[250]	All SBI	7.1	0-60	1.08 (1.04 – 1.11)	0.77 (0.68 – 0.87)
Manchester triage score	Thompson 2009[249]	'Serious infection'	44.7	3-192	3.40 (2.20 – 5.20)	0.8 (0.8 – 0.9)
	Nijman 2011[251]	All SBI	10.4	1-192	1.35 (1.08 – 1.69)	0.84 (0.73 – 0.98)
Yale Observation score	McCarthy 1982[235]	All SBI	11.9	0-24	3.84 (2.75 – 5.37)	0.15 (0.03 – 0.44)
	Teach 1995[238]	Bacteraemia	2.9	3-36	1.58 (0.85 – 2.93)	0.98 (0.95 – 1.01)
	Galetto-Lacour 2001[189]	All SBI	22.6	0-36	1.60 (0.66 – 3.78)	0.91 (0.74 – 1.12)
	Galetto-Lacour 2003[196]	All SBI	29.2	0-36	1.30 (0.58 – 2.92)	0.93 (0.74 – 1.18)
	Thayyil 2005[101]	All SBI	11.1	1-36	2.70 (1.72 – 4.13)	0.19 (0.03 – 1.17)
	Hsiao 2006[197]	All SBI	10.3	2-6	1.10 (0.62 – 1.98)	0.97 (0.82 – 1.15)
	Andreola 2007[191]	All SBI	23.0	0-36	1.80 (1.38 – 2.35)	0.68 (0.55 – 0.85)
SBI score (various)	Bleeker 2001[255]	All SBI	25.1	1-36	5.97 (3.08 – 11.54)	0.66 (0.54 – 0.81)
	Bleeker 2007[97]	All SBI	26.0	1-36	2.07 (1.72 – 2.51)	0.38 (0.27 – 0.55)
	Brent 2011[9]	All SBI	3.8	1-180	11.0 (5.33 – 22.7)	0.86 (0.78 – 0.85)
	Nijman 2013[96]	Pneumonia	14.5	1-180	11.1 (6.57 – 18.7)	0.85 (0.79 – 0.92)
	Nijman 2013[96]	Other SBI	14.5	1-180	9.12 (5.54 – 15.0)	0.83 (0.76 – 0.91)
'Lab score'	Galetto-Lacour 2008[7]	All SBI	26.7	0-36	4.66 (3.66 – 6.46)	0.07 (0.02 – 0.21)
	Galetto-Lacour 2010[253]	All SBI	22.7	0-36	5.09 (3.93 – 6.59)	0.17 (0.10 – 0.28)

Table 8: Performance characteristics of risk prediction scores for the diagnosis of SBI in children presenting to the ED. LR+: Positive likelihood ratio, LR-: Negative likelihood ratio. LRs were calculated using the epiR package in R[100].

1.7. Summary

Acute febrile illnesses are a common presentation to the children's Emergency Department. A failure to recognise SBI in febrile children and initiate prompt therapy is associated with adverse outcomes. Conversely, the inability to rule out SBI in a majority of febrile children results in the overtreatment of self-limiting illnesses. The consequences of this approach are an excess of hospital admissions, and an overuse of antibiotics, associated with the development of antibiotic resistance.

Serious bacterial infections caused by vaccine-preventable organisms are in decline, and the aetiology of SBI is evolving as a consequence. The changing aetiology of the most invasive infections, such as bacteraemia, may further challenge the ability of clinicians to recognise and initiate treatment with effective antibiotics.

Improved diagnostic testing using clinical or biomarker variables, and combinations thereof, may improve the assessment of risk of SBI, rationalising decision-making with regard to empirical treatment and hospital admission. Molecular methods of pathogen identification may also speed the identification of causative organisms and guide definitive antimicrobial therapy.

1.8. Hypothesis

The prediction of SBI in febrile children of all ages presenting to the ED can be improved by the combination of clinical and biomarker variables in risk prediction models.

1.9. Aims and objectives

a) To describe the changing aetiology of bacteraemia presenting to the children's ED, and to examine the impact of aetiology on clinical recognition of disease.
b) To evaluate the use of a universal 16S rRNA PCR with sequencing method for the diagnosis of bacteraemia in the children's ED.
c) To derive and internally validate a clinical predication rule for the diagnosis of SBI in the children's ED, incorporating recently described biomarkers of SBI.
d) To externally validate the performance of published prediction rules in a prospectively recruited sample of children presenting to the ED with acute febrile illness, and to estimate the improvement in diagnostic accuracy obtained by the addition of multiple biomarkers.

Chapter 2: Methods

2.1. Overview of the chapter

The work presented in this thesis is the result of the analysis of two large datasets designed to describe the aetiology of bacteraemia and other Serious Bacterial Infection (SBI) presenting to the children's Emergency Department (ED), and to evaluate the diagnostic accuracy of molecular tests, clinical and biomarker variables for the diagnosis of SBI in this setting. In this chapter I will set out the methods used in the large, prospective diagnostic accuracy study which provided the context for the work. Though detailed statistical methods are located in the relevant results chapters, in this chapter I also detail conventional methods for quantifying diagnostic accuracy.

2.2. Setting

All of the work presented was undertaken in the Alder Hey Children's Hospital NHS Foundation Trust ED. This is the busiest children's ED in the UK, with approximately 60 000 attendances per year. Alder Hey is situated in Liverpool and provides a range of secondary and tertiary paediatric services to children throughout the North West of England. It has been the focus of research into Meningococcal Disease (MCD) for over 20 years.

2.3. Contributors

Professor Enitan Carrol developed the study protocol for the Salivary Procalcitonin in Children presenting to the Emergency Department (SPICED) study and secured funding from the National Institute for Health Research (NIHR). As my PhD supervisor, Professor Carrol supported me to identify further funding from the Alder Hey Children's Charity and the University of Liverpool in order to carry out further biomarker analysis, and the evaluation of a broad-range molecular diagnostic, SepsiTest.

The work benefitted greatly from a highly motivated clinical staff working in the ED, and a long established commitment to clinical research in the department, overseen by Dr Omnia Marzouk, a co-investigator on the SPICED study. Guidance was given by the Steering Committee, Drs Paul McNamara, Ruwanthi Kolamunnage-Dona, and Paul Newland, and Ms Kim Williams, Ms Rachel Breen, Ms Sarah Olsen, Ms Jenny Newman, and Ms Dot Lambert. I undertook the day to day management of the SPICED study, establishing the mechanisms for recruitment into the study, communicating with and providing feedback to clinical staff, and revising the initial protocol and case report form (CRF) into the final versions used. Together with the research nurses Ms Alison Grant and Ms Rhian Williams, we directly recruited children in to the study, and having organised the provision of Good Clinical Practice training in the department, supported clinical staff to appropriately recruit to the study throughout the working week. This research team completed data entry onto paper CRFs. Together with Dr Duncan Appelbe, I developed and revised a Microsoft Access database, and I was responsible for data entry into the database, and for the cross checking of all CRFs. I was responsible for liaising with the UK Clinical Research Network (UKCRN) Coordinating Centre, and uploading recruitment data on a monthly basis. Outcome diagnoses for children recruited to the SPICED study were attributed by Professor Carrol, Dr Stephane Paulus and I.

Clinical samples obtained in the ED were prepared and stored with the help of the Biochemistry and Microbiology departments at Alder Hey, and in particular Ms Christine Chesters, Ms Elaine Hanmer and Ms Teresa Barton. Procalcitonin (PCT) measurement was performed on the B.R.A.H.M.S Kryptor platform (Thermofisher scientific, Hennigsdorf) and was carried out by Ms Chester. I was supported in the performance of Neutrophil Gelatinase-associated Lipocalin (NGAL) and Resistin analysis by Mr Graham Jeffers, while the performance of the broad-range molecular test (SepsiTest) was undertaken by the Molzym laboratory in Bremen, with the oversight of Professor Michael Lorenz.

Statistical analysis was undertaken with the support of Professor Peter Diggle. I also received training from Drs Rob Christley and Jon Read as part of the Advanced

Statistics for Epidemiology course at the University of Liverpool, and from Ms Sally Eagle who together taught me to programme using the R statistical package.

The study of bacteraemia was conceived by Dr Richard Drew, Professor Carrol and I. Informatics support was provided by Ms Laura Medway and Ms Gemma Boydell who exported data from the Alder Hey Trust microbiology and biochemistry databases. I was supported in the process of data entry and cleaning by the Research Fellow Dr Kha Nguyen, and by a group of final year undergraduate medical students who I co-supervised as part of a Paediatric Infectious Disease 'Selective in Advanced Medical Practice'.

2.4. The Salivary Procalcitonin in Children presenting to the Emergency Department (SPICED) study

2.4.1. Background

The measurement of biomarkers in saliva is a non-invasive approach to diagnostic testing which has particular appeal in the study of children. Saliva has been demonstrated to be an appropriate sample for the measurement of protein biomarkers, as well as molecular diagnostics, and salivary biomarkers may reflect both local and systemic disease[256]. Salivary Procalcitonin has previously been detected in adults with diabetes and periodontitis[257].

2.4.2. Objectives

The primary objective of the SPICED study was to evaluate the diagnostic accuracy of Procalcitonin, measured in saliva samples, for the diagnosis of SBI in febrile children presenting to the ED. Recognising the limitations of individual markers of SBI in children, and building upon earlier work which identified markers of SBI in Malawian children[215], the study incorporated the measurement of NGAL and Resistin in blood samples, and the comprehensive collection of clinical data with which to evaluate multivariable risk prediction models. The study also provided the infrastructure in which to evaluate the molecular diagnostic SepsiTest in whole blood in children.

2.4.3. Study design

The SPICED study was a prospective observational diagnostic accuracy study of febrile children presenting to the Emergency department. It was funded by the NIHR Research for Innovation, Speculation and Creativity stream (Project ID: RC-PG-0309-10053).

2.4.4. Identification of eligible patients

Children less than 16 years of age with fever (>38°C) or history of fever were eligible for inclusion in the study if they required blood tests as part of routine clinical management. At triage, nursing staff were prompted by an electronic triage screen to record a 'fever, or history of fever'. Children who met this criterion received a SPICED study 'tag' which was attached to the clinical notes. Simultaneously they were electronically flagged to the study research team. At clinical review, if the attending clinician decided to undertake blood tests, the research team would approach the child and family with written information relating to the study (see Appendix 1 for an example of age appropriate patient information provided to eligible children).

All documentation required to recruit children to the study was included in numbered study packs which were available in the busy clinical observation area of the ED.

2.4.5. Exclusion criteria

Children with an underlying immunodeficiency or with significant periodontal disease were excluded.

2.4.6. Ethics approval

Written informed consent was obtained from those with parental responsibility for the study subjects (Appendix 2). Where appropriate in older children, the assent of study subjects was also recorded. Ethical approval for the study was granted by the Greater Manchester West Research Ethics Committee (10/H1014/53), and site approval granted by the Alder Hey Children's NHS Foundation Trust Research and Development department. Specific ethical approval was obtained for a process of

deferred written consent whereby in the context of an acute clinical emergency, or on occasions where the attending clinical team felt unable to give adequate information to allow parents to make an informed decision, parents were able to give verbal consent for clinicians or study staff to store excess clinical samples. Following a later discussion regarding the objectives of the study, parents could give written consent for their child to be included, and for the stored clinical samples to be salvaged for analysis

2.4.7. Timescale

Recruitment to the study commenced in November 2010 and was completed in April 2012.

2.4.8. Data collection and storage

Potential markers of SBI were identified from published data[10,185]. The full list of clinical and biomarker variables investigated is detailed in the pre-designed CRF (Appendix 3). Clinical data were entered onto CRFs at or shortly after the clinical assessment. Where possible, this was done by the attending clinician. When clinical information was not fully entered onto the CRF at the time of assessment, the missing clinical information was taken from the clinical notes, where explicitly referenced. Paper CRFs were collected by the study team on a daily basis, and stored in a locked office. All data recorded on the CRFs were cross checked against the clinical notes which had been electronically scanned and stored.

2.4.9. Data entry

Data from the paper CRFs were transcribed into a password protected Microsoft Access database. Information stored electronically on Trust databases (such as demographic data, admission and discharge dates, and results of blood tests) was imported directly into the study database. A random selection of approximately 10% (120) of CRFs was cross checked against the electronic record to identify transcription errors. None were identified.

2.4.10. Standards for Reporting of Diagnostic Accuracy (STARD)

The study was reported in line with the STARD initiative guidelines, detailed in the Introduction (Section 1.5.1).

2.4.11. Outcome diagnoses

Outcome diagnoses were determined independently by Professor Carrol, or another Paediatric Infectious Disease Consultant (Dr Paulus) and I, according to pre-determined criteria (Table 9). These criteria were agreed with reference to previously published diagnostic accuracy studies of SBI in children [9,10,185,253]. Outcome diagnoses were blinded to the results of the biomarkers analysed (PCT, NGAL and Resistin), though not to the clinical information including routine clinical tests. Routine clinical tests were requested at the discretion of the clinical team, and not all tests were performed in all children. In the event of disagreement, a final outcome diagnosis was attributed by consensus with the involvement of all three investigators. Subjects were followed up to 28 days post discharge to minimise the risk of misclassification.

Diagnosis	Criteria
Pneumonia	Respiratory symptoms and signs and focal consolidation on X-ray reported by a paediatric radiologist
Other SBI	
Bacteraemia	Identification of a significant bacterial pathogen in blood using culture or molecular methods,
Urinary tract infection	Growth of a single bacterial urinary tract pathogen at $\geq 10^5$ colony-forming units/ml in an appropriate urine sample in the context of clinical signs of systemic involvement.
Meningitis	Identification of a bacterial pathogen in CSF using culture or molecular methods, or clinical meningitis plus a cerebrospinal fluid polymorphonuclear leucocytosis
Osteomyelitis	Clinical signs, and radiological confirmation or identification of a pathogen in the bloodstream
Septic arthritis	Isolation of a bacterial pathogen from a joint
Probable bacteraemia	Identification of a plausible pathogen, in the context of a compatible clinical syndrome and/or clinical signs of bacteraemia (see Table 10 and below for definition of clinical signs)
Probable SBI	Prolonged admission and administration of intravenous antibiotics beyond 72h despite negative culture results.

Table 9: Pre-defined criteria for the diagnosis of SBI[9,10,185,253]

'Clinical signs' of bacteraemia were considered to be signs of sepsis on presentation (including fever, tachycardia, tachypnoea, poor perfusion), associated with elevated markers of infection (White Cell Count and C-Reactive Protein) and the prolonged administration of intravenous antibiotics, and prolonged hospital stay despite negative culture results. Supportive microbiological evidence of infection was also considered, such as a serological response to a clinically plausible organism (for example an elevated anti-Streptolysin O titre in a clinical presentation consistent with a Streptococcal infection). Criteria for the outcome of bacteraemia are detailed in Table 10.

Culture	Specific PCR	SepsiTest	Clinical signs	Additional microbiology	Outcome
Pathogen	±	±	±	±	Bacteraemia
±	Pathogen	±	±	±	Bacteraemia
±	±	Pathogen	±	±	Bacteraemia
Possible pathogen:					
Single isolation	-ve	Same organism	±	±	Bacteraemia
	-ve	-ve	+ve	±	Probable bacteraemia
	-ve	-ve	-ve	±	No bacteraemia
Repeated isolation	-ve	±	±	±	Bacteraemia
Negative	-ve	Possible pathogen	+ve	±	Probable bacteraemia
			-ve	±	No bacteraemia
Negative	-ve	Negative	+ve	+ve	Probable bacteraemia
			-ve	±	No bacteraemia

Table 10: Outcome diagnosis of bacteraemia according to microbiological and clinical evidence

2.5. Laboratory methods

2.5.1. Sample collection and preparation

Alongside routine clinical samples, study samples were collected by trained clinical or research staff using a conventional aseptic non-touch technique.

2.5.1.1. Plasma

A sample of 0.5-1.0ml of blood collected into Lithium Heparin was transported to the laboratory at ambient temperature, and centrifuged at 13,000 rpm for 5 minutes using a Heraeus Biofuge Pico centrifuge. The plasma was removed and stored in Sarstedt 0.5ml microtubes at -80°C. Sample freezing occurred within 1 hour. Samples were tracked using the hospital laboratory tracking system.

2.5.1.2. EDTA

A sample of 0.5-1.0ml of whole blood was collected into paediatric EDTA bottles. Samples were inverted at the bedside to prevent clotting. In the microbiology laboratory, samples were transferred in sterile conditions into DNA-free Universal Microbe Detection (UMD) tubes containing glycerol (Molzym, Bremen). These were then vortex mixed for 15 seconds. Samples were labelled with a study ID and stored at -80°C until analysis.

2.5.1.3. Saliva

Saliva samples were collected using plain cotton swab 'Salivettes' (Sarstedt, Numbrecht). Children were encouraged to chew or suck the swab for 45 seconds as described in the product instructions for use. The swab was then returned to the Salivette container and centrifuged for 2 minutes at 13 000 rpm, allowing the collection of the saliva sample in the extended tip of the collection chamber. Up to 1ml of saliva was stored at -80°C until analysis. Sample freezing occurred within 1 hour, and samples were tracked using the hospital laboratory tracking system.

Prior to analysis the samples were thawed, mixed thoroughly by vortex mixer and centrifuged again to remove bubbles and particulate matter.

2.5.2. Enzyme-linked immunosorbent assay (ELISA)

The ELISA is a well –established method for quantifying an unknown substance in clinical samples. The general principle involves the specific binding of antibody to the antigen of interest, followed by an enzyme reaction causing a detectable signal (such as a colour change). In the sandwich ELISA technique, the antigen of interest in the sample is bound first to antibody on a pre-treated plate (the 'capture antibody'), and this bound antigen is then 'sandwiched' by the addition of a second, complementary detection antibody to which an enzyme is conjugated. Following the addition of the enzyme substrate the resultant colour change may be quantified by an absorbance reader. Accurate quantification of the antigen requires the production of a standard curve. For this, absorbance readings from samples with a known concentration of antigen ('standards') are used to plot the curve that relates absorbance to antigen concentration for each assay. Absorbance readings taken from clinical samples can then be applied to the standard curve for that assay to allow the calculation of the antigen concentration.

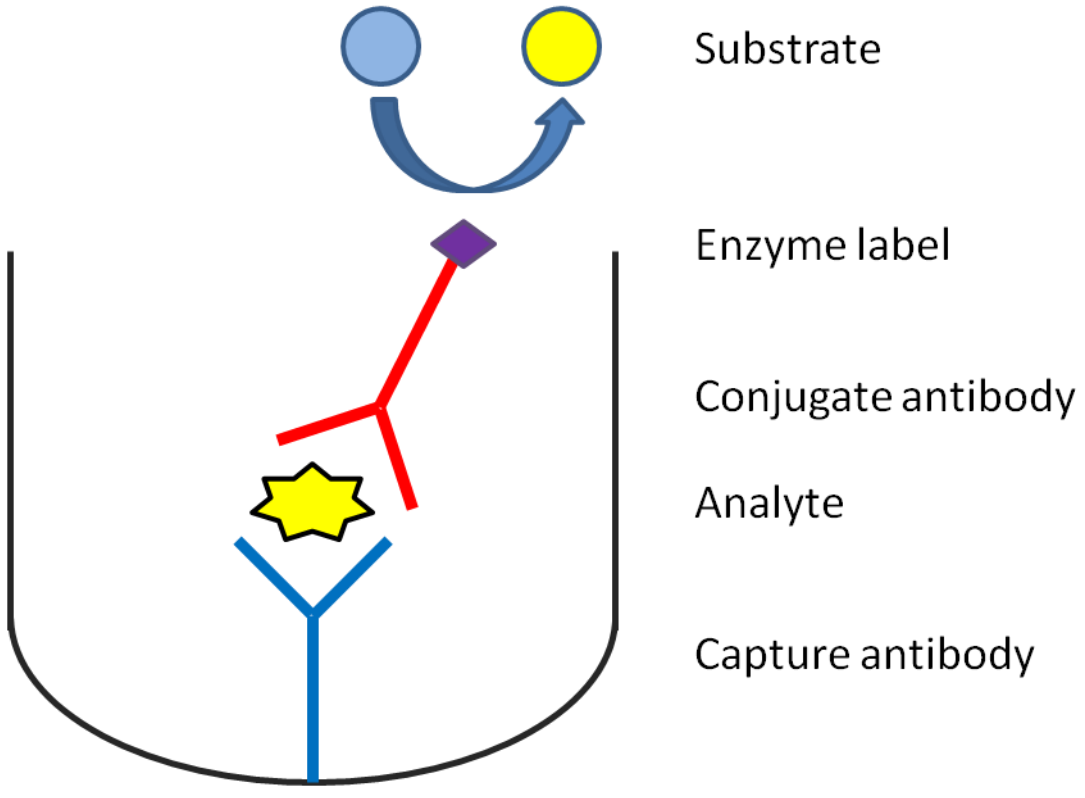


Figure 3: Schematic of a simple sandwich ELISA assay.

2.5.2.1. Neutrophil Gelatinase-associated Lipocalin (NGAL)

Measurement of NGAL was undertaken using a commercially available ELISA, validated for use on plasma samples (R&D systems, Minneapolis) according to the supplied protocol.

2.5.2.1.1. Reagent and sample preparation

A serial dilution of the supplied human NGAL standard was prepared following reconstitution of the standard solution (100ng/ml) with deionised water. Standard concentrations ranged from 0.156ng/ml to 10ng/ml. The supplied wash buffer, calibrator diluents, and substrate solutions were prepared according to the instructions provided.

Plasma samples were initially diluted 1:20 as per the protocol by mixing 20µl of plasma with 380 µl of calibrator diluent. In view of the high number of samples exceeding the upper limit of detection, subsequent assays were run at a dilution of 1:50 (20µl plasma in 980µl diluent).

2.5.2.1.2. Assay procedure

The assay was performed on the supplied pre-coated 96-well NGAL microplate. 100µl assay diluent was added to each well, before the addition of 50µl of prepared standard or sample. Initially, assays were undertaken in duplicate (n=80) and intra-assay variability was measured by estimating the coefficient of variation (CV). For the NGAL assay CV was estimated to be 4.16%. Following the demonstration of adequate intra-assay precision, later samples were assayed individually.

Standards and samples were incubated for 2 hours at 4°C before washing with the supplied wash buffer using an autowasher. 200µl human NGAL conjugate (stored at 4°C) was then added to each well, and incubated for a further 2 hours at 4°C. Following a further wash cycle, 200µl substrate solution was added to each well and incubated in a dark room at room temperature for 30 minutes. Stop solution was then added to each well, and the optical density read by the ELx800 absorbance reader (Biotek instruments, Winooski) set to 450nm. A 4 parameter logistic curve

was constructed with each assay run, using the KC junior software package produced by Biotek.

2.5.2.2. Resistin

Plasma samples were analysed using a commercially available, validated Resistin ELISA (Assaypro, Missouri) according to the manufacturer's instructions.

2.5.2.2.1. Reagent and sample preparation

A serial dilution of the supplied human Resistin standard was prepared following reconstitution of the standard solution (16ng/ml) with deionised water. Standard concentrations ranged from 0.25ng/ml to 8ng/ml. The supplied wash buffer, calibrator diluents, and substrate solutions were prepared according to the instructions provided. Plasma samples were diluted 1:20 by mixing 20µl of plasma with 380µl of calibrator diluent.

2.5.2.2.2. Assay procedure

The assay was performed on the supplied pre-coated 96-well Resistin microplate. 50µl of prepared standard or sample was added to each well. Duplicate assays (n=80) again demonstrated acceptable intra-assay variability (CV 3.91%), so subsequent biomarker measurement was undertaken on individual samples.

Standards and samples were incubated for 2 hours at room temperature before washing with the supplied wash buffer using an autowasher. 50µl of biotinylated Resistin antibody was then added to each well and incubated for a further 2 hours. Following a further wash cycle, 50µl of Streptavidin-Peroxidase conjugate was then added to each well, and incubated for a further 30 minutes. After washing, 50µl of Chromogen Substrate was added and incubated for approximately 12 minutes, tapping the microplate to ensure mixing. Stop solution was then added to each well, and the optical density read by the ELx800 absorbance reader (Biotek instruments, Winooski) set to 450nm. A 4 parameter logistic curve was constructed with each assay run, using the KC junior software package produced by Biotek.

2.5.3. Automated Procalcitonin assay (Kryptor)

2.5.3.1. Assay procedure

PCT analysis in plasma and saliva was undertaken on the B.R.A.H.M.S. Kryptor platform (Thermofisher scientific, Hennigsdorf) in the biochemistry department of the Alder Hey Children's Hospital NHS Foundation Trust. The samples were analysed according to manufacturer's instructions, in batches of 50. Quality control samples were analysed at the beginning and end of each run.

2.5.4. SepsiTest 16S rRNA PCR with sequencing

Frozen samples stored in UMD tubes were shipped in dry ice to be analysed by Molzym in Bremen.

2.5.4.1. Assay procedure

Thawed samples were analysed using the semi-automated broad-range real-time PCR, SepsiTest (Molzym, Bremen). The kit includes all reagents for automated DNA isolation and universal 16S and 18S rRNA PCR analysis of bacterial and fungal DNA, respectively. Amplifications were performed in a DNA Engine Opticon cycler (BioRad, Hercules, USA) following the recommended programme in the SepsiTest manual.

2.5.4.2. Sequencing of amplicons

In case of positive reactions, amplicons were sequenced as advised in the manual using supplied sequencing primers. Standard precautions for the avoidance of air and handling-borne DNA contamination were followed. Sequence chromatograms were carefully analysed and sequences were edited manually if necessary. Strains were identified using NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and SepsiTest-BLAST (http://www.sepsitest-blast.net). A result was recorded negative if the melt curve analysis of the real-time PCR indicated absence of bacteria and fungi-specific peaks, respectively, or sequencing did not give a read-out. At sequence identities >97 and ≥99% genus and species names were filed, respectively. Mixed sequences (i.e., chromatograms showing overlapping

sequences of two or more different sequences) were analysed using the Ripseq mixed programme (Isentio, Bergen). Cases positive in the PCR assay and with mixed sequences that could not be resolved were assigned positive for bacteria.

2.5.5. Routine microbiology and virology investigation

Clinical tests were requested at the discretion of the clinical team. All samples were processed in Clinical Pathology Accredited (CPA) laboratories. Blood (0.5 to 1ml) was inoculated into blood culture bottles and monitored using the BacT/ALERT 3D system (Biomerieux, Marcy l'Etoile). Positive blood cultures were processed in line with the UK standard for Microbiology investigations developed by Public Health England[258].

Molecular identification of *Streptococcus pneumoniae* and *Neisseria meningitidis* was performed using specific PCR assays at the Meningococcal Reference Unit in Manchester[259,260]. Multiplex viral PCR was performed on respiratory (Respiratory Syncytial Virus, Influenza A and B, Parainfluenza 1, 2 and 3, Adenovirus, Rhinovirus and Metapneumovirus) and CSF (Herpes Simplex, Varicella Zoster and Enterovirus) samples in the regional virology laboratory at Manchester and subsequently the Royal Liverpool Hospital.

Urinalysis was performed in the ED using Multistix 8 reagent strips (Siemens, Erlangen). Samples of urine and CSF underwent conventional microscopy and culture on agar gel plates, and were processed at Alder Hey in line with the relevant UK Standard for Microbiology Investigations[261,262].

2.6. Statistical methods

Statistical analysis was undertaken using the R statistical package[263]. Each chapter includes a detailed description of the statistical methods employed.

2.6.1. Regression analysis

The regression methods described here are drawn from the book "Regression modelling strategies" by Frank Harrell[264] and course notes from the "Advanced Statistics for Epidemiology" MSc module led by Dr Rob Christley at the University of Liverpool.

Regression analysis is a statistical method used to describe relationships between dependent (outcome) and independent (predictor) variables. Using sample data, a model relating outcome to a combination of explanatory predictors can be estimated.

2.6.1.1. Linear regression

The simplest such model is the linear regression model:

$$y = \alpha + \beta x$$

where y is the outcome of interest, x are the observed values of an explanatory variable, and α and β represent unknown 'parameters' estimated to best fit the data. To reflect variability in the outcome variable, this mathematical model becomes the statistical model:

$$Y_i = \alpha + \beta x_i + Z_i, \; i=1....n$$

The statistical model includes the term Z_i which quantifies the amount each measured x_i deviates from the straight line. This is the 'error' or 'residual' term. i are the number of measured variables in the dataset, hence the ability to estimate the model parameters depends upon the size of the dataset, n.

Several assumptions are necessary for linear regression. These are that the residuals are an independent random sample (implying that the observations are independent), from a normal distribution with a mean of zero (the relationship between Y and x is linear) and constant variance. Under these assumptions, maximum likelihood estimates can be made for the parameters α and β. That is that, given the data, these are the parameter estimates that make the data most likely.

The simple model relating outcome to a single explanatory variable can be extended to include more than one. The inclusion of multiple variables may improve the fit of the model to the data, helping to explain more of the variation in the observed outcome. It also allows an exploration of the effect of each variable in the model, whilst controlling for that of the other variables.

2.6.1.2. Non-linear explanatory variables

One of the key assumptions of linear regression is that the residuals have a mean of zero, and that the relationship between explanatory variable and outcome is linear. It may be apparent in the initial data exploration that this is not true. Various approaches to these non-linear associations are possible, including the use of polynomial and piecewise transformations of the explanatory variable.

2.6.1.2.1. Polynomial transformations

In settings where the assumption of linearity appears not to hold, alternative relationships can be explored by the use of polynomial terms, e.g.

$$Y= \alpha + \beta_1 x+ \beta_2 x^2+ \beta_i x^i + Z_i,\ i=1....n$$

Though the relationship between Y and x is non-linear, the regression function remains linear in terms of the unknown parameters (α , β), and so polynomial models are considered an example of multivariable linear regression. Maximum

likelihood estimates for the unknown parameters can be made by considering each higher order polynomial as independent variables in the regression model.

2.6.1.2.2. Piecewise transformations

Instead of considering a non-linear relationship between outcome and explanatory variable as a polynomial function, non-linear variation can be viewed as a combination of more than one adjacent linear relationship. This use of more than one linear segment may allow the model to fit the data more closely. Identifying the most appropriate 'breakpoint' – the point at which the slope of the line changes in order to best fit the data – can be achieved statistically, that is, by iterating over all possible breakpoints to find the model which best fits the data, or mechanistically by choosing a breakpoint which explains an expected variation in the model.

2.6.1.3. Generalised linear models

So far, the methods described have maintained the necessary assumptions of normally distributed residuals (there is a linear relationship between explanatory variables and outcome), and of constant variance. However, in a number of situations these assumptions are clearly false. In the course of this thesis there are two such examples. These are the analysis of data where the outcome variable is binary (or categorical), and the analysis of count data.

Generalised linear models (GLMs) relax the requirement for outcome and explanatory variable to be directly linear. Instead a GLM has the following properties to relate outcome to predictors:

- an error structure
- a linear predictor
- a link function

These properties will be illustrated with reference to the common GLMs used in the thesis.

2.6.1.3.1. Logistic regression

Logistic regression may be used when outcome data are binary. In this setting, the relationship between outcome and explanatory variables is clearly not linear (the outcome is either 0 or 1). This is the context for much of the work of this thesis, where the outcome of interest is the presence or absence of disease (SBI). Even when the outcome is expressed as a probability of SBI (p), a simple linear regression would predict probabilities that are impossible (outside the range 0 to 1). Further, the variance of a binomial distribution (a distribution of the probability of obtaining a 1, where the data may be 0 or 1) varies (variance is proportional to p(1-p)), breaching the assumption of constant variance.

Logistic regression overcomes these limitations of linear regression. As described, logistic regression as an example of a GLM specifies an error structure, and a link function which allows the predicted value of the outcome to be derived from the linear predictor – the linear sum of the effects of the explanatory variables.

For logistic regression, the link function g(Y) is the logit function, ln (p/1-p). So,

$$g(Y) = \ln(p/1-p) = \alpha + \beta_1 x_{1i} + \beta_2 x_{2i} + \beta_p x_{pi} + Z_i,\ i=1....n$$

The error structure specific to logistic regression limits the residual terms Z_i to the binomial distribution.

The results of a logistic regression model then can conveniently be transformed into the probability of the outcome of interest. From the equation above, the probability of the outcome can be written:

$$p = e^{g(Y)}/1+e^{g(Y)}$$

with g(Y) calculated from the particular set of explanatory variables in the model.

2.6.1.3.2. Poisson log-linear regression

The second example of a GLM used in the thesis is in the case of count data. For count data with frequent zero counts, the variance of the data is assumed to equal the mean, and thus increases with the mean (it is not constant). Once again, the error structure is non-normal, as count data are bounded by zero (it is not possible to have a negative count). In this setting, the link function is the log function (which ensures that data are bounded below), while the error structure follows the poisson distribution.

2.6.1.3.3. Generalised additive models

Generalised Additive Models (GAMs) are a form of GLM which provide a valuable method to explore the relationship between a non-linear explanatory variable and outcome. GAMs do not assume a linear relationship but instead apply non-parametric smoother functions to local subsets of data[265] and thus can describe complex non-linear relationships. For the purpose of this thesis, the use of GAMs has been limited to providing an illustration of the likely shape of the relationship between outcome and explanatory variable, in order to identify appropriate non-linear relationships (such as piecewise or polynomial transformations) which better model the data.

2.6.2. Imputation of missing data

In the analysis of large datasets with multiple variables, missing data is a common problem. The handling of missing data has significant implications for the conclusions drawn from the data. The appropriate response to the problem of missing data depends upon the pattern of missingness. Where the probability of missingness of a given variable is independent of both the observed variables, and the unobserved, missing variables, the data is said to be missing completely at random (MCAR). This is an unusual case. More commonly, it is plausible to assume that the missingness depends upon observed variables, termed missing at random (MAR). This MAR assumption may be particularly appropriate where there are many predictor variables[266]. Where the probability of missingness is dependent

upon the missing variables, the problem is termed 'non-ignorable' or missing not at random (MNAR). The methods used to make efficient, unbiased use of incomplete data depend upon these assumptions[267].

2.6.2.1. Complete case analysis

Analysis of only those cases in which data are completely recorded is both inefficient, as well as almost certainly leading to biased inferences. Only where the MCAR assumption holds does a complete case analysis yield unbiased inferences. The use of complete case analysis in datasets with many variables is inefficient as only those cases in which all variables of interest are complete can be included.

2.6.2.2. Ad-hoc methods

Ad-hoc methods include a variety of methods which either recode missing variables to some common value (such as in mean imputation), or drop those variables with a high proportion of missing values. Like complete case analyses, such approaches are generally considered to lead to biased inferences, and inefficient use of the available data[268].

2.6.2.3. Multiple imputation

Multiple imputation is a statistical method in which missing data are replaced by random draws from a distribution of plausible values. The generation of plausible values requires the appropriate specification of an imputation model relating missing data to that of the observed data. For this model to be appropriate it should preserve both the structure of the observed data, and the uncertainty underlying it. From this distribution of plausible values, multiple imputations are made resulting in the generation of multiple, 'completed' datasets. These multiple datasets are analysed for the outcome of interest, and the results of each analysis pooled. The resultant pooled analysis generates estimates whose standard errors reflect the uncertainty inherent in the missing data[269].

2.6.2.4. Likelihood based methods

In maximum likelihood methods, multiple imputations of missing values are entered, alongside the probability of obtaining each value, given the observed data.

This ‘augmented’ dataset is then used to undertake the regression analysis, accounting for the weight of each possible imputed value[267].

2.6.3. Statistical measures of diagnostic accuracy

2.6.3.1. Evaluating dichotomous test results: the 2x2 table

The diagnostic accuracy of a test – a clinical variable, a biomarker, or a prediction model – evaluates the agreement between the test and the outcome of interest, as determined by the reference standard. Tests should ideally be as ‘sensitive’ and ‘specific’ as possible. Sensitivity is the proportion of individuals with the outcome of interest identified by a positive test, whilst specificity is the proportion of individuals without the outcome correctly identified by a negative test[270]. These, and other measures of the value of a test, can be illustrated by a ‘2x2 table’ (Table 11) relating test outcomes to the ‘true’ diagnosis, in this case of SBI.

		SBI	
		+	-
Test	+	a	b
	-	c	d

Table 11: A 2x2 table relating test results to outcome diagnosis (SBI).

Sensitivity	=	a / a+c
Specificity	=	d / b+d
Positive predictive value	=	a / a+b
Negative predictive value	=	d / c+d
Positive likelihood ratio	=	True positive rate / false positive rate (a / a+c) / 1-(d / b+d)
Negative likelihood ratio	=	False negative rate / true negative rate (1-(a / a+c)) / (d / b+d)

The *sensitivity* and *specificity* of a test do not identify the likelihood that a subject with a positive test has the outcome of interest. This is described by the positive predictive value. Positive and negative predictive values are however, highly dependent upon the prevalence of the outcome. If the outcome of interest is rare, positive predictive values of sensitive and specific tests will remain low, whilst poor tests will achieve a high negative predictive value[271].

Likelihood ratios are the ratio of the probability of a given test result in a subject with the disease to that of a subject without the disease. The likelihood ratio allows the calculation of post-test odds from pre-test odds. Hence a discriminatory test will have a positive likelihood ratio significantly higher than 1, and a negative likelihood ratio significantly less than 1[272]. Various authors have suggested positive and negative likelihood ratios that respectively rule in and rule out diagnoses. In the diagnosis of SBI, a test with a positive likelihood ratio of more than 5 is proposed as a 'red flag' test capable of ruling in SBI, whilst one with a negative likelihood ratio of less than 0.2 is proposed to effectively rule out SBI[8].

2.6.3.2. Evaluating diagnostic risk prediction models

An established approach to evaluating the diagnostic accuracy of multiple variables for a binary outcome is multivariable logistic regression. A logistic regression model provides a predicted probability of a binary outcome of interest for given values of the explanatory variables. For each threshold value of the predicted probability, the outcome may be dichotomised and summary statistics such as sensitivity, specificity, predictive values and likelihood ratios calculated as described above. A change in the threshold to increase sensitivity invariably reduces specificity, and various methods exist to 'optimise' this threshold value. Studies of biomarkers of SBI in children have commonly achieved this using the 'Youden index' which sums sensitivity and specificity[273]. This approach assumes equal weighting for positive and negative outcomes, and has been criticised. Increasingly, authors are identifying multiple thresholds at which important clinical decisions to rule in or rule out SBI can be made[185].

A number of measures of the overall performance of logistic regression models exist. Broadly, these measures quantify the distance between predictions and observed outcomes, with better fitting models minimising this distance[274]. The most commonly reported summary statistic of a logistic regression model is the concordance (*c*) statistic. The *c* statistic is the likelihood that a randomly selected subject with the outcome of interest will have a higher predicted probability than another randomly selected subject who does not have the outcome[275]. It is the overall measure of discrimination of the model. Conveniently, in logistic regression, the *c* statistic is equal to the area under the Receiver Operating Characteristic (ROC) curve (AUC) which plots sensitivity against (1-specificity) for each probability of an outcome. Though rarely undertaken, it is equally plausible to create ROC curves to summarise positive and negative predictive probabilities.

Another important consideration in the evaluation of a logistic regression model is the calibration between predictions and observed outcomes. In simple terms, in a well calibrated model a predicted risk of 10% should result in 10 outcomes of interest per 100 subjects with this risk. This can be represented by a calibration plot. Subjects with similar predicted probabilities are grouped together and mean predictions plotted against observed outcomes. When subjects are grouped into deciles, this is a graphical representation of the Hosmer-Lemeshow 'goodness of fit' test[274].

2.6.3.3. Evaluating improvements in risk prediction models

The increasing acceptance of risk prediction models in the context of diagnostics has prompted recognition of the need to evaluate improvements of one model over another. Quantifying improvement in traditional measures of model performance, such as the AUC, may be of value, but has been shown to be difficult. The addition of variables with a significant association with outcome to a baseline model with reasonable discrimination, may fail to produce a meaningful increase in the AUC. Such an increase often requires the addition of variables with unrealistically high effect size[276].

Alternative approaches have been proposed. One attempts to quantify the improvement in classification achieved by the assignment of subjects into risk categories[277]. Improved classification occurs when subjects with the outcome of interest are re-classified into higher risk categories. Worse classification occurs when the new model re-classifies a subject with the outcome to a lower risk category. The converse is true for subjects without the outcome of interest and so reclassification is outcome specific. The Net Reclassification Improvement (NRI) is defined as the sum of differences of proportions of subjects with the event moving into a higher risk category minus those moving into a lower risk category, and of those without the event who move into a lower risk category minus those who move into a higher risk category. NRI provides a clinically relevant measure to evaluate the relative performance of two models[277].

Use of the NRI in this way assumes the existence of clinically meaningful risk categories. In cardiovascular disease clinical guidelines incorporate established risk thresholds to guide treatment decisions[278], but in many clinical situations, including in febrile illness in children, this is not the case. In this situation, the identification of risk categories must be justified. Alternatively, the 'category-free' (or continuous) NRI eliminates the need for risk categories and defines any increase in probability in the new model as upward reclassification. The category-free NRI equals twice the difference in the probabilities of upward classification in events, minus non-events[276].

Chapter 3: Bacteraemia in the Alder Hey Children's Hospital NHS Foundation Trust Emergency Department 2001-2011

3.1 Overview of the chapter

This chapter describes the analysis of all positive blood cultures isolated from children presenting via the Alder Hey Children's Hospital Emergency Department between 2001 and 2011.

3.2 Background

3.2.1 Bacteraemia presenting to the children's Emergency Department

Bacteraemia is estimated to occur in approximately 1 in 250 febrile children under the age of 5 years presenting to the ED[6]. Although relatively uncommon, bacteraemia is associated with significant morbidity and mortality. Meningococcal disease (MCD) remains the most common infectious cause of childhood death outside the neonatal period[13]. Prior to the introduction of the Pneumococcal vaccine in the UK, an estimated 20% of childhood mortality was infection related, with 'septicaemia' the most commonly documented cause of death[37].

3.2.2 Aetiology of paediatric bacteraemia in the UK

The aetiology of paediatric bacteraemia in the UK has evolved following the introduction of effective vaccines against the important childhood pathogens *Streptococcus pneumoniae, Neisseria meningitidis* and *Haemophilus influenzae*. Rates of admission to hospital with septicaemia caused by these organisms have substantially declined[12]. Over the same period, the recorded incidence of childhood bacteraemia caused by Gram negative infections (such as Enterobacteraeciae) and of Gram positive infections such as Staphylococci and non-pyogenic Streptococci has increased[40].

A recent study of septicaemia and meningitis in hospitals in south London estimated that two thirds of 'community-acquired' septicaemia or meningitis in 2009-2011 – that is, septicaemia and meningitis diagnosed within 48 hours of presentation to hospital –occurred in children with significant comorbidities. In children with no comorbidities, the estimated incidence of community-acquired septicaemia was 5.3 per 100 000[39].

3.2.3 Categorising paediatric bacteraemia

Historically, serious infections such as bacteraemia have been categorized by the timing of their identification into 'community-acquired' and 'hospital-acquired' infection. These categories were used to inform the likely aetiology, and guide empirical therapy. There is an increasing awareness of patients who, though they acquire serious infections in the community, share the characteristics of patients with 'hospital-acquired' infections. Such infections have been termed 'healthcare-associated'[279]. Adult studies have indicated that healthcare-associated Gram negative bacteraemia is associated with delays in the administration of appropriate antibiotic therapy when compared with community-acquired infections[280]. To date no validated definition of healthcare-associated bacteraemia is in use in children.[281]

3.2.4 Timely antimicrobial therapy in paediatric bacteraemia

Timely, effective antimicrobial therapy alongside supportive therapy is fundamental to the management of serious infections in children. The Surviving Sepsis campaign recommends the administration of antibiotics within one hour of recognition of severe sepsis[41]. Delays in the recognition of MCD[14], in administering antibiotics, and suboptimal resuscitation of septic shock contribute to mortality in children[15,16].

3.2.5 Appropriate empirical antimicrobial therapy

Presently it is not possible to confirm the presence of, still less the aetiology of bacteraemia in the ED. Empirical antimicrobial therapy then is initiated prior to confirmation of infection, and must be based upon the likely causative organism, and an understanding of local susceptibilities of these organisms. Unsurprisingly, in order to improve outcomes, empirical antimicrobial therapy needs to be effective. In a large multicentre study of adults with bacteraemia in Spain, inadequate empirical antimicrobial therapy – therapy to which the isolated organism was not susceptible *in vitro* - was associated with increased mortality[282]. Another large retrospective analysis of 5715 adults with septic shock demonstrated that inadequate empirical antibiotics resulted in a 5 fold increase in mortality[283].

3.3 Hypothesis

The evolving aetiology of paediatric bacteraemia may impact the ability of ED clinicians to recognise the diagnosis and to initiate timely, effective antimicrobial therapy.

3.4 Aims and objectives

This study set out to describe the aetiology of childhood bacteraemia presenting to the Alder Hey Children's Hospital NHS Foundation Trust ED between 2001 and 2011. Specifically, the study objectives were to:

I. Report temporal trends in the aetiology of bacteraemia in the Alder Hey Emergency Department between 2001 and 2011
II. Describe the clinical characteristics of children presenting to the Alder Hey Emergency Department with bacteraemia between 2001 and 2011
III. Explore the impact of temporal trends in aetiology upon the timeliness and appropriateness of empirical antibiotic therapy

3.5 Methods

3.5.1 Study design

This was a retrospective times series analysis of all clinically significant episodes of culture-positive bacteraemia presenting to the Alder Hey Children's Hospital ED.

3.5.2 Data collection

All positive blood culture isolates recorded in the laboratory database between 2001 and 2011 inclusive were identified. Isolates were excluded if they were not associated with a presentation to the ED within the 48h prior to the sample being taken.

Clinical data relating to each episode of bacteraemia was extracted from clinical notes, and from the Hospital Trust electronic database. Data was recorded on demographics and significant comorbidities, including the presence or absence of a central venous line (CVL) or other prosthetic material. Commonly used markers of infection (White cell count, Neutrophil count, Platelets and C-reactive protein) were extracted electronically. Antibiotic susceptibility data on all significant isolates was retrieved from the microbiology database.

In order to examine trends and to compare our data with published evidence, we grouped episodes of *Streptococcus pneumoniae, Neisseria meningitidis* and *Haemophilus influenzae* as 'vaccine-preventable' infections, and similarly grouped 'Gram negative' infections. For the remaining Gram positive infections we differentiated between typically pathogenic organisms in healthy children such as *Staphylococcus aureus* ('typical Gram positive'), and those considered to be associated with the healthcare setting such as Coagulase-negative staphylococcus ('other Gram positive').

3.5.3 Definitions

Community-acquired (CA) bacteraemia was defined as the identification of a significant pathogen in a blood culture taken within 48 hours of presentation to the ED, in the absence of admission to hospital in the previous month, and in the absence of any features of healthcare-associated bacteraemia.

Healthcare-associated (HCA) bacteraemia was defined as the identification of a significant pathogen in a blood culture taken within 48 hours of presentation to the ED, in the following children:

- those with an indwelling device (such as CVL, ventriculo-peritoneal shunt or other prosthetic material)
- those with primary or acquired immunodeficiency
- those requiring regular hospital based intervention (such as haemodialysis, or intravenous therapies)
- pre-term infants less than 12 months from discharge from the neonatal unit

Hospital-acquired bacteraemia was defined as the identification of a significant pathogen in blood culture in a child who had been admitted to hospital in the previous month. Children with hospital-acquired bacteraemia were excluded from the analysis.

The identification of on organism classified as a common commensal according to the US Centers for Disease Control, were considered contaminants unless cultured on more than one occasion within 48 hours[284].

Empirical therapy was defined as therapy instituted prior to microbiological evidence of infection, limited to 24 hours from presentation to the ED. Therapy instituted beyond this period, or where there was documentation of microbiological advice was termed ‘directed’.

Susceptibility to empirical therapy was defined as susceptibility to the empirical therapy initiated by the clinical team. Susceptibility was determined by breakpoints in use at the time of isolation, according to the British Society for Antimicrobial Chemotherapy (BSAC) methodology.[285]

A significant comorbidity was any diagnosis requiring the ongoing care of a hospital paediatrician.

3.5.4 Statistical methods

Statistical analysis was undertaken in R, version 3.0.1.[263]

3.5.4.1 Summary statistics

Continuous data were described by median and interquartile range, and comparison between groups performed using the Kruskal-Wallis test. Categorical variables were expressed as percentages with 95% confidence intervals (95% CI) and groups compared by means of a χ^2 test of association. In order to compare uncommon events between groups (such as death), a Monte Carlo simulation was used to check the accuracy of the asymptotic χ^2 approximation to the null sampling distribution.

3.5.4.2 Time series analysis

Poisson log-linear regression was used to model weekly counts of bacteraemia using the glm() function in R, with weekly ED attendance as an offset. To test for over-dispersion in the data, an additional dispersion parameter was included in the model. Time, and a sine-cosine variable representing seasonal variation with an annual period, were included as covariates. In each case, the time series of residuals was examined for evidence of auto-correlation using the acf() function in R (see Figure 4 and Figure 5).

Logistic regression, again implemented in the glm() function in R, was used to model the probability over time that each clinical episode occurred:

1) in a child with significant comorbidities

2) in a child with an indwelling CVL

3) with an isolate that was susceptible to empirical antibiotic therapy.

Scatter plots and generalised additive model (GAM) plots[286] of explanatory variables, fitted using the gam() function in the mgcv R package, were examined for evidence of non-linearity on the log-odds scale.

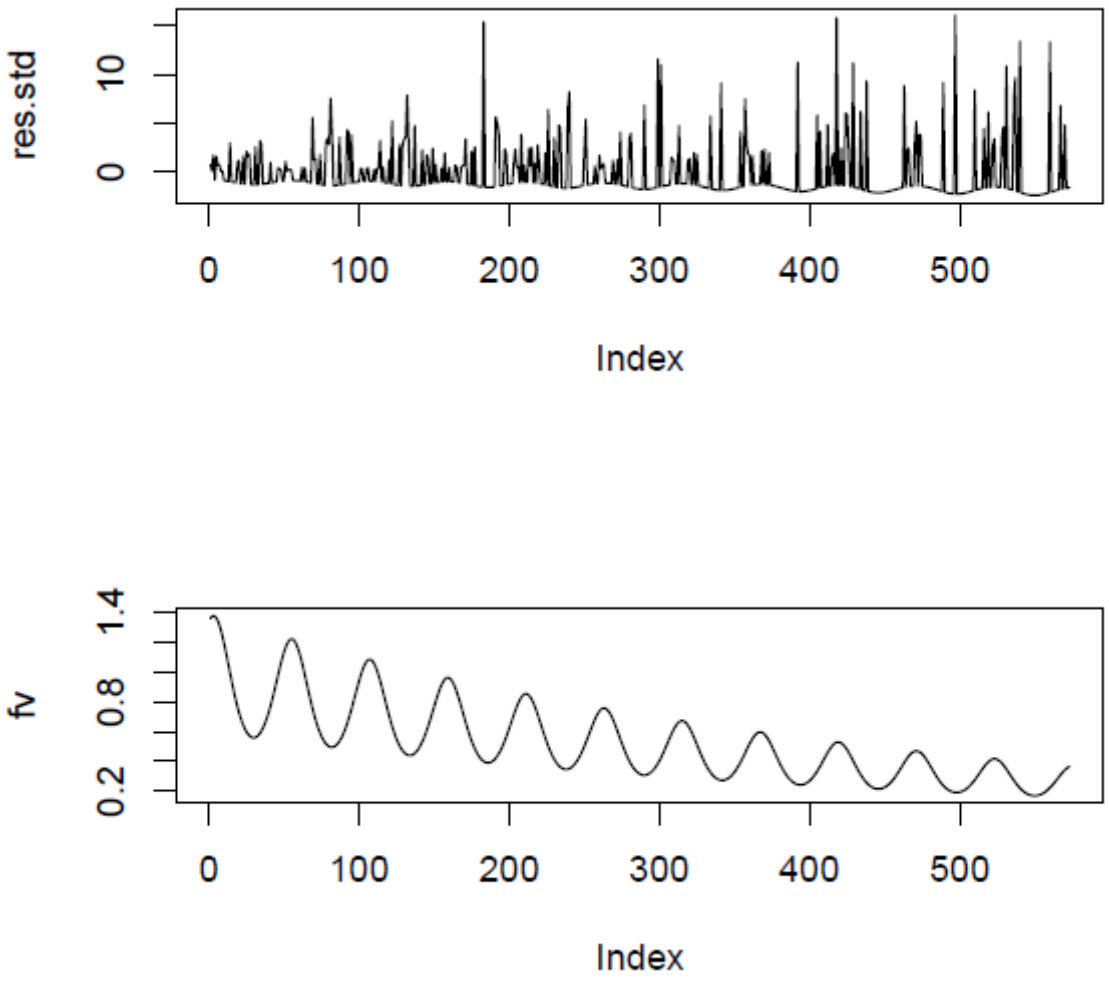


Figure 4: Standardised residuals ('res.std', top) and fitted values ('fv', bottom) for the modelled vaccine preventable bacteraemia data

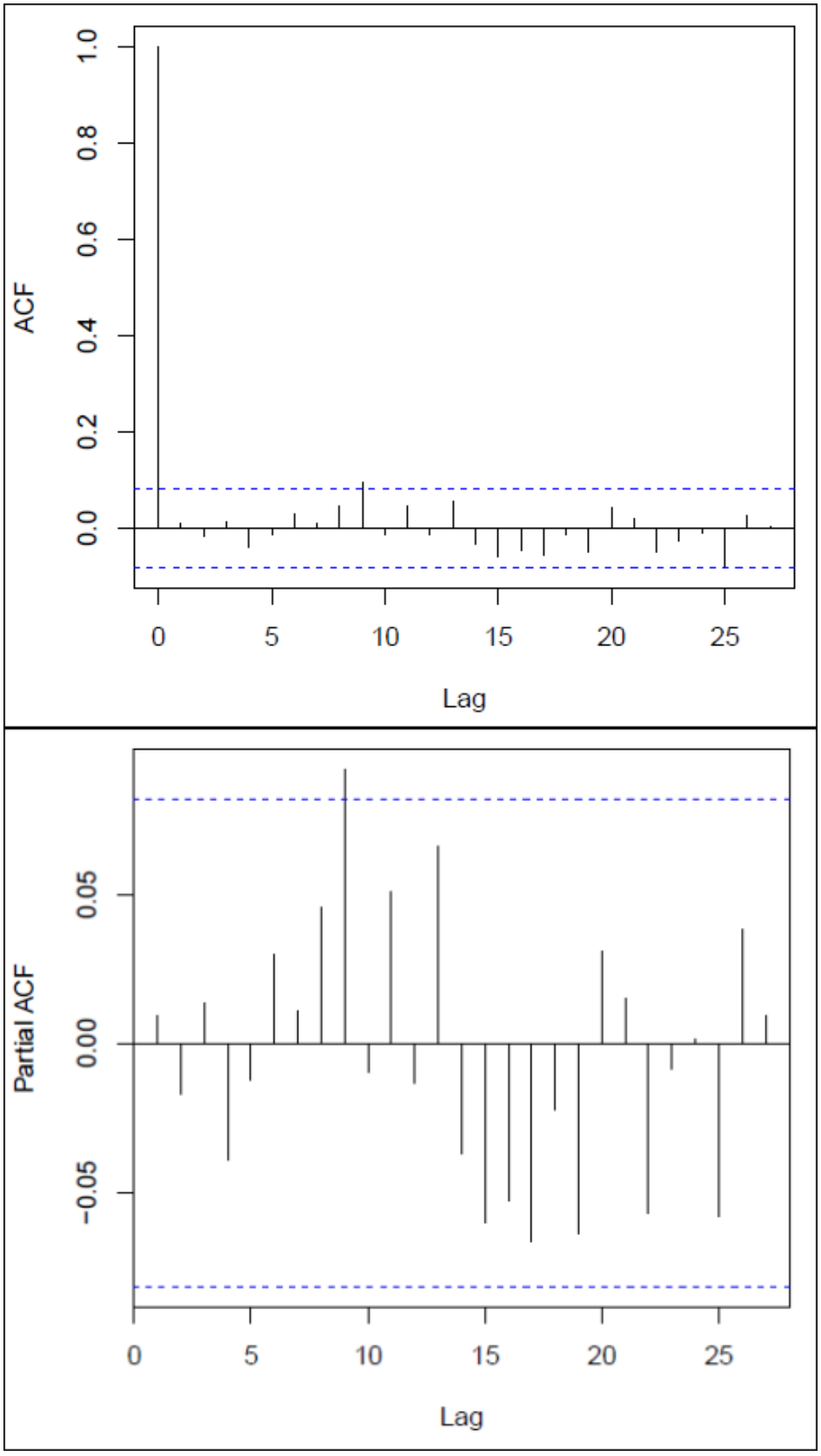


Figure 5: Autocorrelation (top) and partial autocorrelation (bottom) functions of the time series model describing vaccine-preventable bacteraemia. Dashed lines are point-wise 95% limits for uncorrelated residuals.

In the CVL model, inspection of the GAM plot suggested an initial increase in the likelihood of a clinical episode occurring in a child with an indwelling CVL, and subsequent reduction from 2008. In parallel, there was an initial reduction in the likelihood of an isolate being susceptible to empirical antibiotics, with an increase after 2008 (Figure 6). Late in 2007 the hospital increased investment in specialist intravenous nurses, with responsibility for training in the management of CVLs. We considered this a plausible explanation of the observed variation, and implemented a piecewise fit of time with the breakpoint specified to occur at the time of the clinical intervention. Inclusion of the piecewise variable improved the fit of the model to the data (p=0.02, likelihood ratio test).

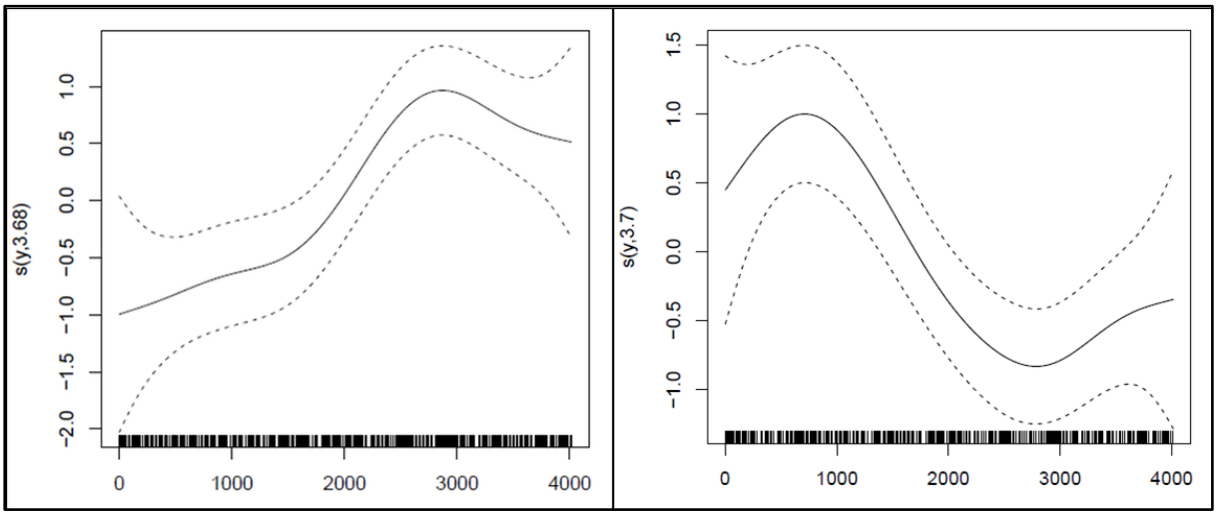


Figure 6: Generalised additive model (GAM) plots of the likelihood of a clinical episode occurring in a child with a CVL *in situ* (left), and of the isolated organism being susceptible to the empirical antibiotic protocol (right). The x axis is time (days from Jan 1st 2001).

3.5.4.3 Multivariable analysis of clinical outcomes

Length of stay (LOS) and time to empirical antibiotics were investigated using multivariable linear regression. Both were log transformed in order to meet the assumption of linearity between outcome and explanatory variables. Plausible explanatory variables (including clinical and biomarker variables) were explored in univariable analyses. Scatter plots and GAM plots were inspected, and correlations between variables explored. In the time to antibiotics model a piecewise fit for platelets was undertaken following inspection of the GAM plot, and found to improve the fit of the model ($p=0.006$, likelihood ratio test). A model including all variables individually significant at the 10% level, i.e. variables with a p-<0.1, was then adopted as the maximal model, and model simplification was undertaken in a backwards stepwise process using the conventional 5% level of significance at each stage.

3.5.5 Ethics approval

The study retrospectively evaluated anonymised data on clinical episodes. Approval for the use of this data was granted by the Research Department of the Alder Hey Children's NHS Foundation Trust.

3.6 Results

Between 2001 and 2011 692 clinically significant blood cultures were identified in children sampled within 48 hours of presentation to the ED. These cultures represented 575 clinical episodes in 525 children. Twenty four children died (4.6%).

3.6.1 Clinical characteristics of children

The majority of clinical episodes occurred in boys (59%, 95% CI 55-63%). Median age at time of presentation was 1.52 years (IQR 0.42-4.27). Figure 7 illustrates the age distribution of children presenting with bacteraemia.

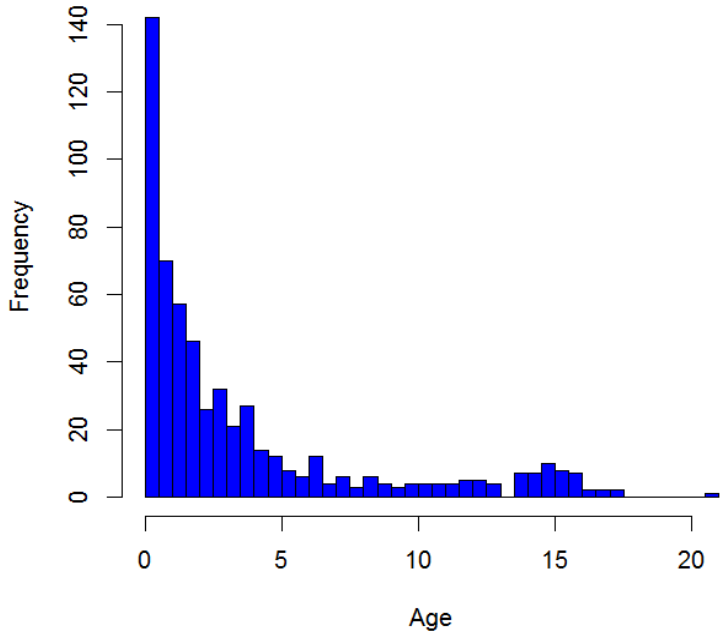


Figure 7: Age distribution of children presenting to ED with bacteraemia.

The characteristics of the 525 children presenting with bacteraemia are summarized in Table 12. Significant comorbidities were present in 151 children (29%). The most common comorbidities were gastrointestinal and the majority of these (23/25) had indwelling CVLs for the purpose of parenteral nutrition (Table 13).

Children with Gram negative isolates were younger, and more likely to have significant comorbidities or an indwelling CVL than those with vaccine-preventable or typical Gram positive isolates. Mortality in children with 'other Gram positive' infections was higher (6/37) than in other groups (OR 5.15, 95% CI 1.60-15.8 versus vaccine-preventable isolates).

	Median	IQR	Vaccine-preventable (n=221)	Typical Gram positive (n=149)	Gram negative (n=118)	Other Gram positive (n=37)	p
Age/years	1.52	0.42-4.27	1.80 (0.83-3.88)	1.58 (0.13-6.82)	0.82 (0.20-3.08)	1.64 (0.69-11.45)	<0.001*
LOS/days	6	3-10	6 (4-9)	7 (3-13)	6 (4-10)	7 (2-11)	0.5*
	%	**95% CI**	**n (%)**	**n (%)**	**n (%)**	**n (%)**	
CM	28.8	25.1-32.8	36 (16.3)	42 (28.2)	51 (43.2)	22 (59.5)	<0.001†
CVL	9.0	6.80-11.7	3 (1.40)	6 (4.02)	26 (22.0)	12 (32.4)	0.001^
PICU	18.9	15.8-22.5	56 (25.3)	23 (15.4)	13 (11.0)	7 (18.9)	0.007†
Mortality	4.57	3.09-6.71	8 (3.62)	6 (4.02)	4 (3.39)	6 (16.2)	0.005^

Table 12: Clinical characteristics of 525 children presenting to the ED with bacteraemia according to type of organism. LOS: Length of hospital stay, CM: Significant comorbidity, CVL: Central venous line *in situ*, PICU: Admission to paediatric intensive care unit. *Kruskal-Wallis †Chi squared.^ Monte Carlo simulation

Comorbidity	Freq	ICD-10 codes
Gastrointestinal	25	K36, K51, K90 Q39, Q42, Q43, D57, Q79
Neurodevelopment	24	G40, G71, Q75 G80 Q02, Q85
Respiratory	15	E84 J45
Renal	13	N04, N13, N18 Q63
Birth/Prematurity	13	PO7, P27
Congenital	11	Q87 Q90
Cardiovascular	11	I34, I45 Q20, Q21, Q25
Haematology/Oncology	10	C40,C56, C85, C91, C96 D43, D66
Skin/Musculoskeletal	10	L20 T84 Q67, Q78
Endocrine/Metabolic	7	E10, E71, E72, E76
Immunodeficiency	6	B20 D82, D83, D84
Rheum	4	M05, M30 Q82
ENT	2	D18 H66
Total	151	

Table 13: Comorbidities by system, with ICD-10 codes

Children with HCA bacteraemia were older than those with CA bacteraemia. LOS in HCA bacteraemia was prolonged by 3.9 days versus CA bacteraemia (95% CI 2.3-5.8 days). Mortality and admission to PICU did not differ between these groups (Table 14).

	CA (n=444)	**HCA (n=81)**	**p**
Median age/years (IQR)	1.43 (0.35-3.88)	2.32 (0.96-6.48)	0.001*
Median LOS/days (IQR)	6 (3-9)	9 (4-18)	<0.001*
PICU/% (95% CI)	19.0 (15.6-22.9)	18.5 (11.6-28.3)	0.15†
Mortality/% (95% CI)	4.1 (2.6-6.3)	7.4 (3.4-15.2)	0.25^
Group			
Vaccine-preventable	207	14	
Typical Gram positive	133	16	<0.001†
Gram negative	82	36	
Other Gram positive	22	15	
Median time to antibiotics /minutes (IQR)	181 (59-321)	218 (91-353)	0.15*

Table 14: Clinical characteristics of Community-acquired (CA, *see definitions*) and Healthcare-associated bacteraemia (HCA). *Kruskal-Wallis †Chi Squared ^Monte Carlo simulation

3.6.2 Aetiology of bacteraemia in the children's ED

Isolated organisms varied with age (Figure 8). In early infancy, the most common organisms were *Escherichia coli* and Group B Streptococcus. There were 47 presentations of neonatal sepsis, including one of early onset disease (Group B Streptococcus). *S.pneumoniae* was the most commonly isolated organism overall. *S.pneumoniae* and *N.meningitidis* occurred commonly between the ages of 1 and 5 years, whilst *S.aureus* was the most common organism in children older than 5 years. Most (87/96) meningococcal isolates were Group B, the remainder were Groups C (5), W135 (3) and Y (1). Two episodes of Methicillin-resistant *S.aureus* bacteraemia occurred over the 11 year period, one associated with ventriculitis in a child with a ventriculo-peritoneal shunt, and one in a previously well child. There were 17 polymicrobial infections in 13 children. Twelve had significant comorbidities and eight had CVLs *in situ*. Isolated organisms are summarized in Table 15.

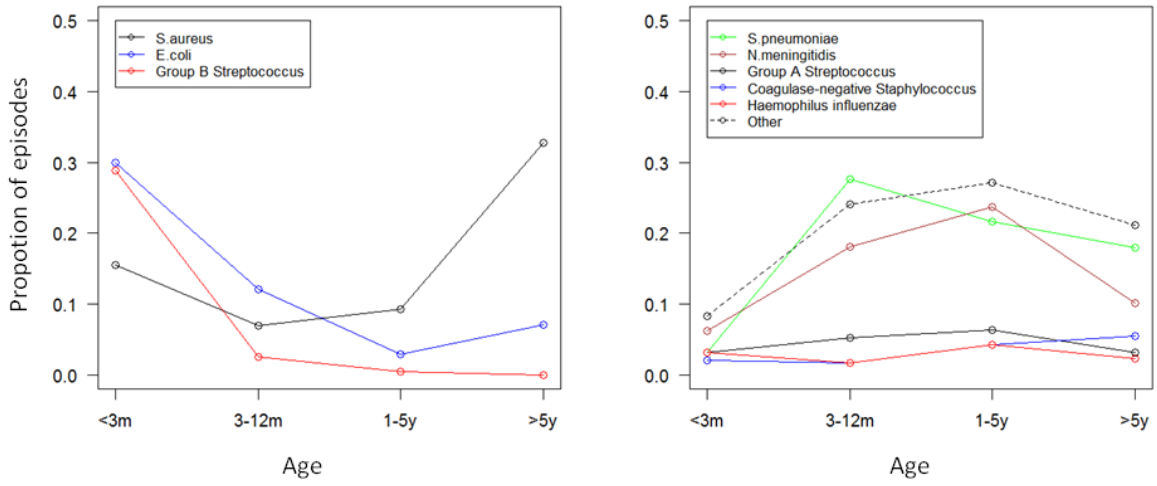


Figure 8: Isolated organisms by age group.

'Vaccine-preventable'	**n=223**	**%**
S.pneumoniae	109	19.0
N.meningitidis	96	16.7
H.influenzae (type B)	18	3.1
'Typical Gram positive'	**n=149**	
S.aureus	89	15.4
Group B Streptococcus	32	5.6
Group A Streptococcus	28	5.9
'Gram negative'	**n=152**	
Escherichia coli	59	10.3
Klebsiella sp.	26	4.5
Acinetobacter sp.	11	1.9
Enterobacter sp.	10	1.7
Pseudomonas sp	10	1.7
Salmonella sp.	9	1.6
Moraxella catarrhalis	9	1.6
Other	18	3.2
'Other Gram positive'	**n=51**	
Coagulase-negative Staphylococcus	21	3.5
Enterococcus sp.	19	3.3
Non pyogenic Streptococcus	6	1.0
Other	5	0.9

Table 15: Predominant organisms by group

3.6.3 Time series analysis

The rate of clinically significant bacteraemia presenting to the children's ED was 1.42 per 1000 attendances (95%CI 1.31 to 1.53). The cumulative frequency plot reveals a declining rate of vaccine-preventable infections including *S.pneumoniae*, and an increasing rate of Gram negative infections (Figure 9).

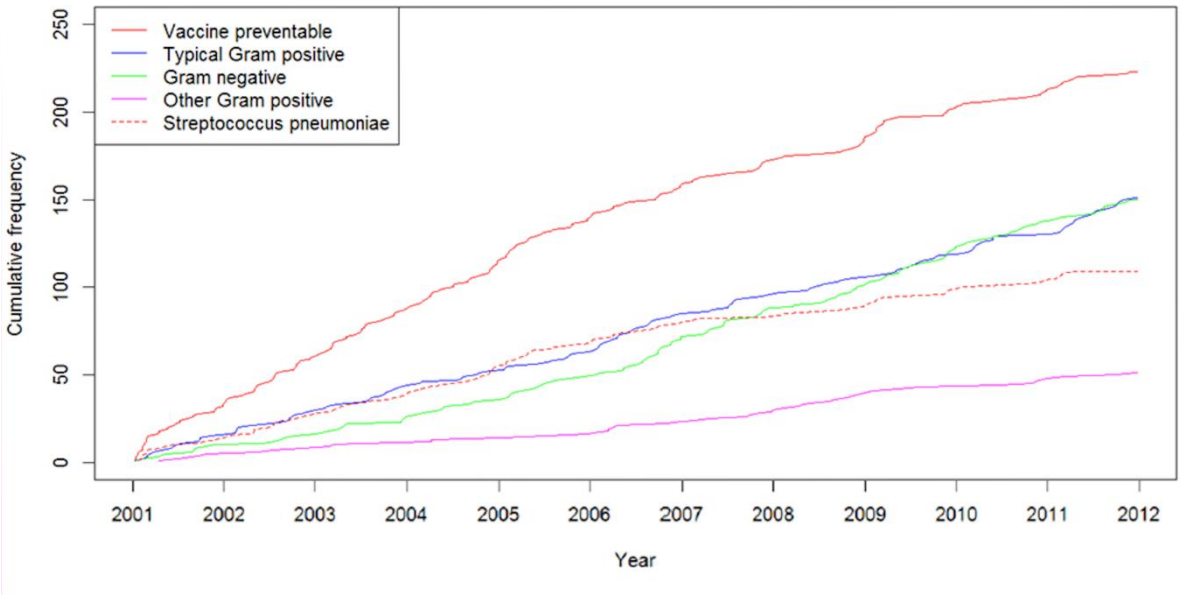


Figure 9: Cumulative frequency plot of bacteraemia presenting to the ED.

Poisson log-linear regression was used to model the observed rate of bacteraemia (Figure 10). For the overall rate of bacteraemia neither the trend over time (p=0.18) nor the seasonal effect (p=0.17) were statistically significant. Bacteraemia caused by vaccine-preventable isolates including *S.pneumoniae* was highly seasonal (p<0.001). From 2001 to 2011 the rate of vaccine-preventable bacteraemia declined from 1.32 to 0.37 per 1000 ED attendances at an annual rate of reduction of 10.6% (95%CI 6.6 to 14.5%). The pneumococcal conjugate vaccine (PCV) was introduced into the UK immunisation schedule in September 2006. When incorporated in the regression model, PCV was associated with a 49% reduction in pneumococcal bacteraemia (95%CI 32 to 74%) from 0.50 to 0.25 per 1000 attendances. By contrast, the rate of Gram negative bacteraemia increased from 0.24 to 0.53 per

1000 ED attendances (p=0.007). The fitted seasonal effect in the Gram negative model, although not statistically significant (p=0.07), showed a peak in summer, in contrast to that of the vaccine-preventable model, which peaked in winter.

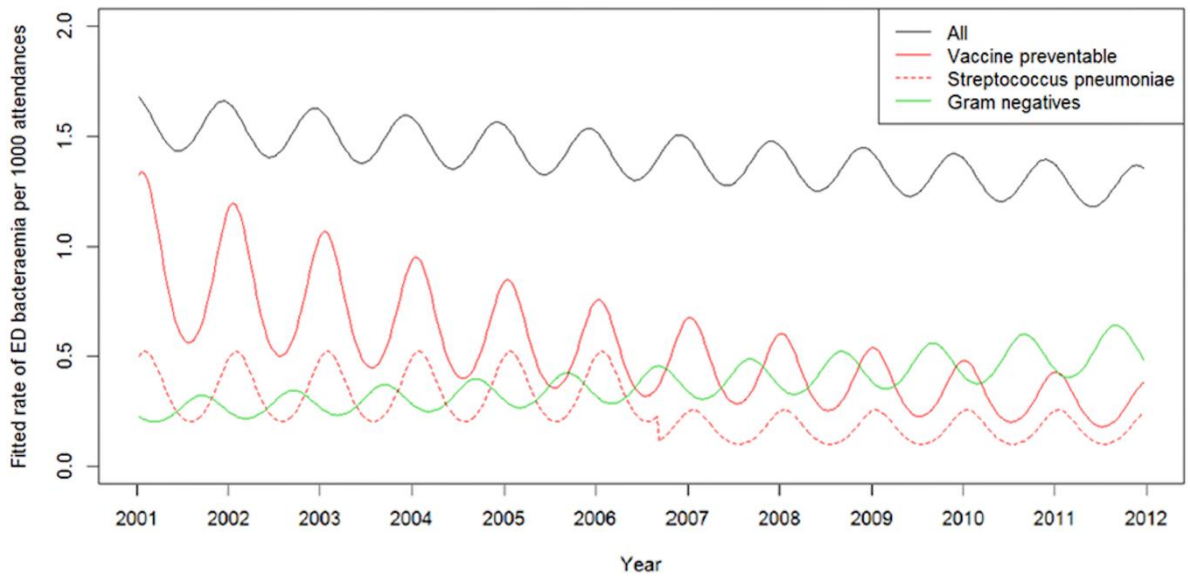


Figure 10: Fitted rate of paediatric bacteraemia per 1000 attendances to the Alder Hey Children's Hospital ED 2001-2011. The discontinuity in the fit for *Streptococcus pneumoniae* is due to the introduction of the PCV in September 2006.

The number of blood cultures requested from the ED was examined as an alternative denominator to that of ED attendance. Only annual data were available. The observed decline in the rate of vaccine-preventable bacteraemia (per 1000 ED attendances) occurred despite an increase in the use of blood culture. The number of blood cultures requested per 1000 ED attendances increased annually by an estimated 7.3 (95% CI 6.0 to 8.6) per 1000 attendances between 2001 and 2011 (Table 16). Following the observation that the number of blood cultures increased over time in parallel with that of Gram negative bacteraemia, we demonstrated an increased rate of Gram negative identifications per 1000 cultures performed (annual increase of 0.17 per 1000 cultures performed, 95% CI 0.02 to 0.32).

16Year	Blood cultures	Annual attendance	Blood cultures / 1000 attendances	Number of Gram negative isolates	Rate of Gram negatives /1000 blood cultures
2001	4864	37051	131.3	11	2.26
2002	4577	35224	129.9	5	1.09
2003	4824	37178	129.8	13	2.69
2004	5517	38092	144.8	11	1.99
2005	5832	38330	152.2	16	2.74
2006	5910	37957	155.7	24	4.06
2007	6626	36944	179.4	25	3.77
2008	6621	37227	177.9	22	3.32
2009	6904	37426	184.5	29	4.20
2010	6846	35969	190.3	22	3.21
2011	6757	35159	192.2	18	2.66

Table 16: Annual rate of blood culture investigations by ED attendance, and rate of isolation of Gram negative bacteraemia

The rate of CA bacteraemia reduced from 1.18 to 0.71 per 1000 ED attendances between 2001 and 2011 (p=0.005), while the rate of HCA bacteraemia increased from 0.18 to 0.50 per 1000 (p=0.002). The proportion of clinical episodes occurring in children with an indwelling CVL increased from 3.2% in 2001 (95% CI 1.47 to 6.84%) to a peak of 26.5% before declining to 21.8% in 2011 (95% CI 12.9 to 34.3%). In parallel, the likelihood that an isolate was susceptible to empirical therapy reduced from 96.3% (95% CI 92.1-98.2%) to 82.6% (95% CI 69.8-90.7) between 2001 and 2011, reaching a nadir of 74.4% in 2008. These trends are illustrated in Figure 11. Neither LOS, likelihood of PICU admission nor mortality changed over time.

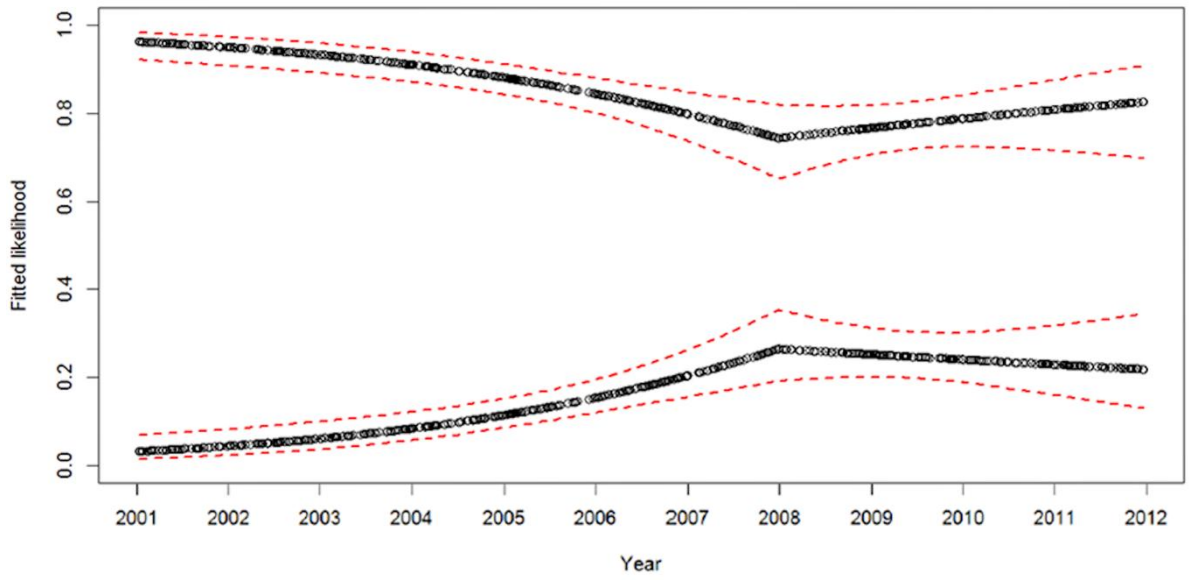


Figure 11: Fitted likelihood over time that an isolate was susceptible to empirical therapy (top), and that an episode occurred in a child with an indwelling CVL (bottom). Time was fit as a piecewise term to incorporate a clinical intervention. Inclusion of the piecewise term improved the fit of the model. Dotted red lines are 95% confidence intervals.

3.6.4 Routine biomarkers in children with bacteraemia

Data on routinely measured blood tests (White cell count, Neutrophil count, Platelet count, and C-reactive protein) were extracted from the Trust database. The range of values in children presenting to the ED with bacteraemia is summarised in Table 17. This study was not designed to evaluate diagnostic accuracy, but it is notable that only half (51%, 280/546) of White cell count (WCC) measurements were outside the range 5-15 x 10^9 cells/litre considered normal by the NICE "Feverish Illness in Children" guideline. Similarly, 41% (227/549) CRP measurements <40mg/dl (a commonly used threshold in diagnostic accuracy studies, see Table 5) were observed in children with bacteraemia. In isolation, CRP and WCC are insufficiently sensitive for the diagnosis of bacteraemia in the ED.

Biomarker	Minimum	Median	IQR	Maximum	n
CRP	0	57.2	13.8-135.6	690.5	549
WCC	0.07	11.66	7.49-19.32	63.95	546
Neutrophils	0.02	7.81	4.29-14.14	49.88	546
Platelets	1	267	189-377	847	546

Table 17: Summary of routinely measured biomarkers in the ED. IQR: interquartile range

Biomarker distribution varied according to the type of organism cultured. Compared to vaccine-preventable bacteraemia, lower values of CRP, WCC and Neutrophil count were observed in typical Gram positive, Gram negative, and other Gram positive infections. Gram negative bacteraemia was associated with a lower Platelet count when compared with vaccine-preventable bacteraemia (Figure 12).

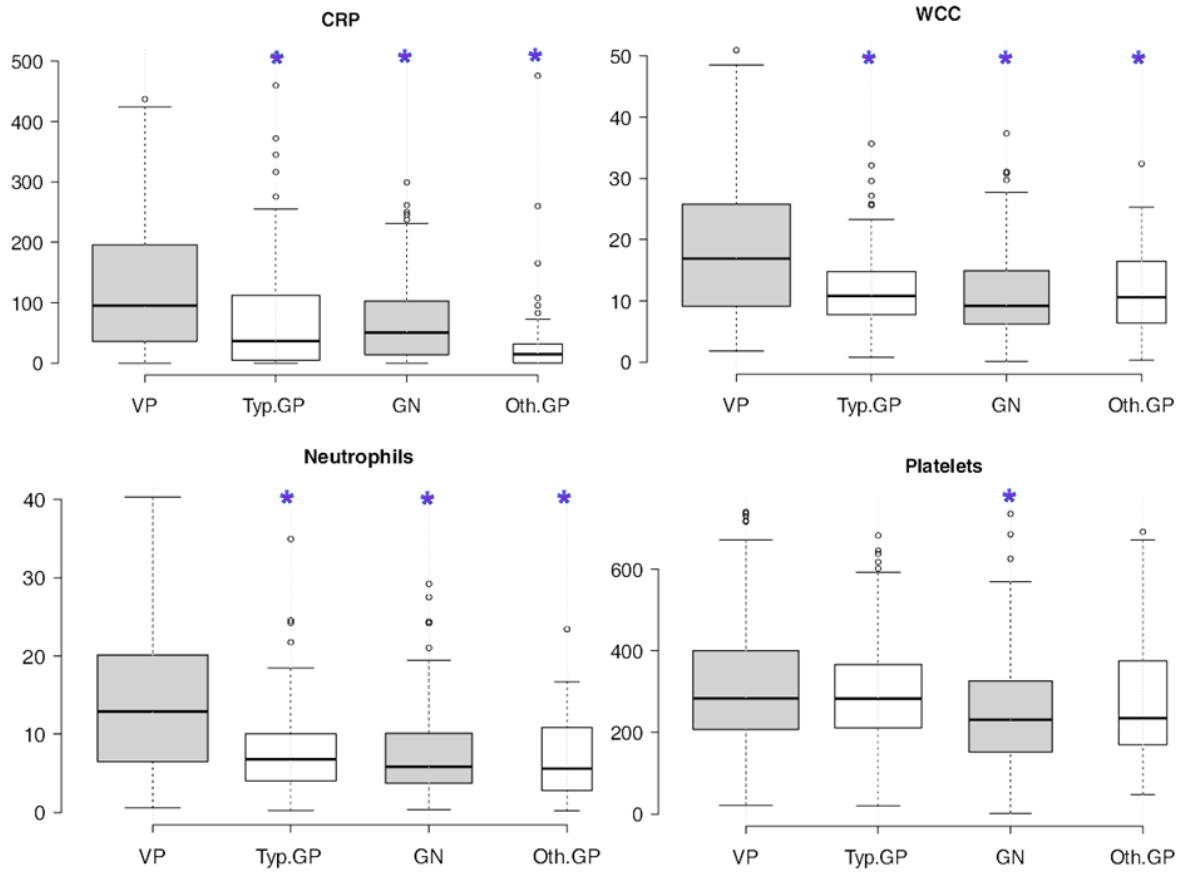


Figure 12: Distribution of biomarkers according to type of isolate. VP: Vaccine-preventable, Typ.GP: Typical Gram positive. GN: Gram negative. Oth.GP: Other Gram positive. Significance tests are comparisons with the Vaccine-preventable group of isolates. *p<0.001

3.6.5 Timeliness and appropriateness of empirical antibiotics

The empirical antibiotic protocol was documented in 563/575 clinical episodes (Table 18). One child died prior to receiving antibiotics, and a further eighteen children received no antibiotics. For children with no indwelling CVL, 375/480 received monotherapy, commonly Cefotaxime (289/375). In combinations, Cefotaxime was also invariably used, alongside Gentamicin, Benzylpenicillin or Amoxicillin. In children with an indwelling CVL, combination therapy was commonly used (46/83), particularly with Teicoplanin and Gentamicin.

No CVL *in situ*		**CVL *in situ***	
Monotherapy		**Monotherapy**	
3rd gen Cephalosporin	295	Cefotaxime	22
1st/2nd gen Cephalosporin	58	Teicoplanin	6
Other	22	Other	8
	375 (78%)		36 (43%)
Combination therapy		**Combination therapy**	
Cefotaxime and Benzylpenicillin	24	Teicoplanin and Gentamicin	27
Cefotaxime and Gentamicin	20	Teicoplanin and Cefotaxime	5
Cefotaxime and Metronidazole	11	Other combination	14
Other combination	32		
	87(18%)		46 (57%)
None	18	None	1
Total	**480**		**83**

Table 18: Empirical antibiotics administered in children with and without CVLs.

It was possible to determine empirical susceptibility in 527 of 575 cases. Vaccine-preventable isolates were almost universally susceptible to empirical therapy (217/219), whilst 22% (29/131) of Gram negative isolates were resistant (p<0.001). Data on time to antibiotics was available for 78% (444/575) of all episodes. Median time to antibiotics was approximately 3 hours, and varied according to the type of organism (Table 19). Vaccine-preventable infections received empirical antibiotics earlier than other types of infection.

	Median /minutes	IQR	p
All	184	62.5-330.5	
Vaccine-preventable	130	34.5-297	
Typical Gram positive	256	118-376	<0.001
Gram negative	184	95.5-356	
Other gram positive	253.5	147-401	

Table 19: Time to antibiotics (minutes) according to type of organism

In order to explore this relationship, a multivariable model of time to antibiotics was developed (Table 20). Following adjustment for other explanatory variables, time to antibiotics was increased by 57 minutes for Gram negative infections compared to vaccine-preventable infections. Older children received antibiotics later than younger children. Overall, time to antibiotics increased by approximately 3 minutes per year of the study (p=0.006 for linear trend).

Both Platelet count and CRP were associated with time to antibiotics. In the case of Platelets, below a value of approximately 250 (as determined by the breakpoint which minimised the residual deviance of the multivariable model), time to antibiotics reduced linearly with the platelet count. Paradoxically, time to antibiotics increased with CRP count. An increase of 100mg/ml was associated with an increased time to antibiotics of 10 minutes.

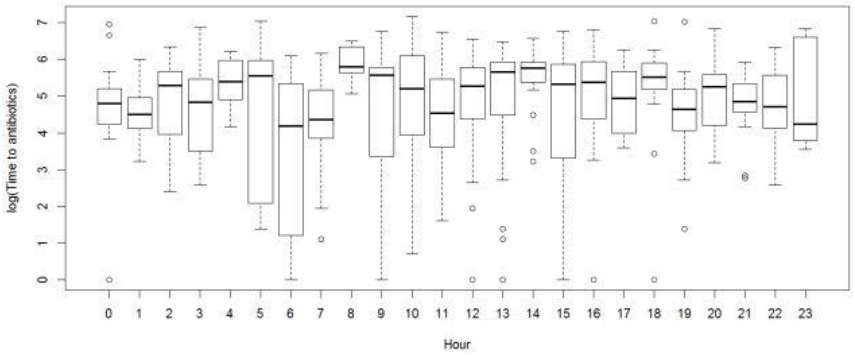
Variable	Estimate	LCI	UCI	p-value
Intercept	3.90	3.20	4.60	<0.001
Group				
Vaccine-preventable	REF			
Typical Gram positive	0.75	0.42	1.07	<0.001
Gram negatives	0.77	0.44	1.10	<0.001
Other Gram positive	1.06	0.47	1.66	<0.001
Age	0.047	0.016	0.078	0.003
Year	0.058	0.017	0.100	0.006
Platelets (left slope)	0.005	0.002	0.008	0.003
Platelets (right slope)	0.0002	-0.001	0.002	0.78
CRP	0.002	0.0008	0.003	0.001

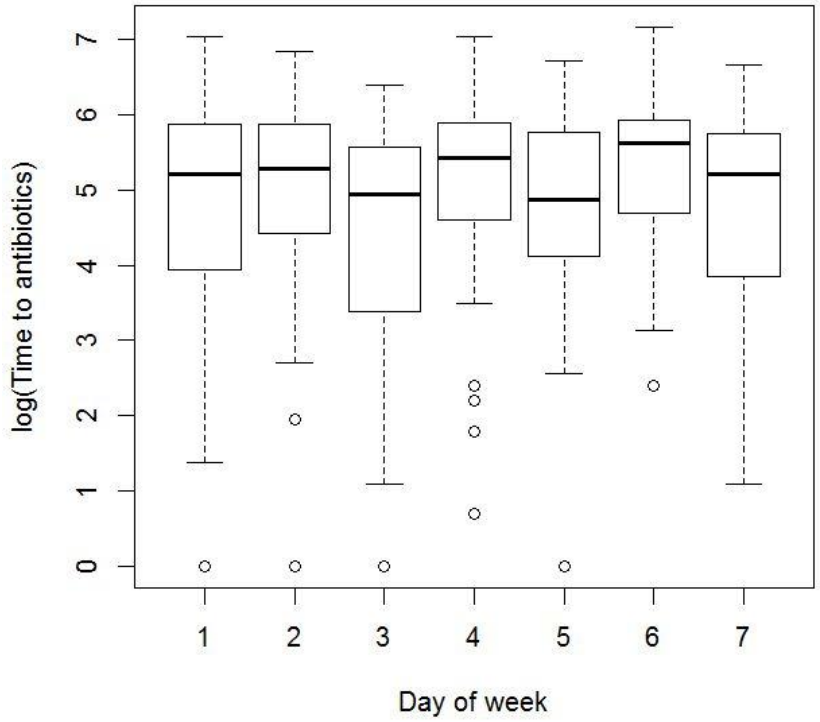
Table 20: Multivariable model of log (time to antibiotics). LCI: Lower confidence interval. UCI: Upper confidence interval.

3.6.6 Time to antibiotics and ED activity

Time to antibiotics is one indicator which is used to evaluate ED 'busyness'. Though no real-time measures of ED activity were available in this retrospective analysis, we examined whether time to antibiotics were associated with the surrogate measures time of day, day of week, month of the year and daily number of ED attendances. When included as categorical variables in regression analysis, there were no significant associations between time to antibiotics and time of day, day of the week or month of the year. These relationships are illustrated in Figure 13. Similarly there was no association of time to antibiotics with daily number of ED attendances.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	4.8914412	0.3401537	14.38	<2e-16***
ED attendance	-0.0003521	0.0031947	-0.11	0.912





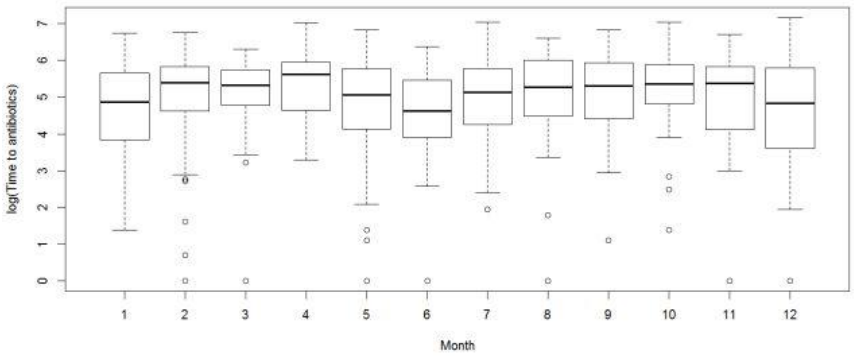


Figure 13: Boxplots of log (Time to antibiotics) against time of day, day of week and month of the year.

3.7 Discussion

This study details the changing aetiology of bacteraemia in the busiest children's ED in the UK. It demonstrates a reduction in the rate of vaccine-preventable bacteraemia, coincident with an increase in Gram negative bacteraemia. The overall rate of bacteraemia is lower than that described in a large study of febrile children presenting to the ED in Australia[6]. This can be explained by the use of a different denominator in this study. As significant bacteraemia presented to the ED in association with a variety of clinical presentations (including bone and joint infections, and abdominal sepsis), the denominator used was all non-trauma presentations. The introduction of the PCV7 in 2006 was associated with a halving of the rate of pneumococcal bacteraemia. These trends are consistent with data from surveillance studies of invasive bacterial infections internationally.[12,39,40,287,288] A seasonal variation was observed in Gram negative bacteraemia with peak in the summer. While not statistically significant this is consistent with other published data.[289,290]

During the course of the study, there was no significant increase in the number of children attending the ED, and ED attendance was used as an offset in the regression modelling to provide rates of bacteraemia per 1000 attendances. There was however a significant increase in the number of blood culture investigations performed in children attending the ED. This increase rules out the possibility that the observed reduction in vaccine-preventable bacteraemia was a consequence of a reduced reliance on blood culture for the diagnosis of bacteraemia. This may have occurred, for example, alongside the increased use of specific, highly sensitive PCR assays. On the contrary, clinicians appeared more inclined to consider bacteraemia, and request blood cultures as time progressed.

Increased rates of blood culture use thus occurred in parallel with the increase in Gram negative bacteraemia identifications. There was a small increase over time in the rate of Gram negative identifications per 1000 cultures performed. The inference that an increased rate of blood culturing may have contributed significantly to the observed increase in the rate of identification of Gram negative organisms – that is, that the increase is not real, but rather reflects increased yield

from a more sensitive sampling approach – seems clinically implausible. Gram negative bacteraemia is a severe infection in children for which prolonged intravenous antibiotics would almost always be considered necessary. The likelihood that despite clinicians undertaking many thousands of negative tests even early in the study period (at which time approximately 1 in 7 of all non-trauma related attendances had a blood culture performed), a significant number of Gram negative isolates were missed appears remote. While we cannot say with certainty that the increased use of blood culture did not contribute to the increase in Gram negative bacteraemia identifications, our data demonstrating an increased rate of Gram negative bacteraemia presenting to the ED are consistent with national laboratory data demonstrating the same[40].

The results illustrate the changing characteristics of children presenting to the ED with bacteraemia. Increasingly these are children with underlying comorbidities, and indwelling CVLs. HCA bacteraemia is of increasing importance. The proportion of episodes involving children with CVLs increased to a peak in 2008 before declining towards the end of the study. This occurred in parallel with an initial reduction in antibiotic susceptibility which also improved after 2008. This is likely to reflect an increased number of children with CVLs receiving total parenteral nutrition (TPN) in the community. Unpublished data from Alder Hey identified a threefold increase in CVL-associated infection rates with the use of TPN (from 3.3 to 10.4 per 1000 line days, personal communication from Sara Melville, specialist vascular access nurse). At the end of 2007, the hospital increased investment in specialist intravenous nurses, with responsibility for training in the management of CVLs. This intervention may explain the later reduction in CVL-associated infections presenting to the ED.

The changing aetiology of childhood bacteraemia in the ED was not associated with temporal changes in mortality or PICU admission. HCA bacteraemia was associated with an increased LOS, irrespective of the responsible organism. Other investigators have related increased LOS in adults to HCA bacteraemia[291] but this has not been described in children before. To date, this category of infection remains poorly defined in children.

Despite acceptance that time to antibiotics influences outcome in sepsis,[292] few studies have reported time to antibiotics in the paediatric ED.[293,294] The results of this study suggest that the changing nature of bacteraemia in the paediatric ED has implications for both recognition and management. A median time to antibiotics of 3 hours is comparable with other published data.[42]

Empirical therapy took longer to initiate in children with Gram negative infections than in children with vaccine-preventable infections, even with adjustment for other explanatory variables. Platelet count and CRP were found to be associated with time to antibiotics. It is unlikely that the tests were being used to guide treatment, rather that the tests were indicative of a more general clinical condition which prompted therapy. The association of a low platelet count with a shortened time to antibiotics is intuitive, as thrombocytopenia is a recognised feature of severe sepsis. The association between CRP and increasing time to antibiotics is a little more difficult to explain however. It is well recognised that CRP is insensitive in the early stages of severe infections[191], and so a plausible explanation is that those most fulminant presentations which prompted early antibiotic administration were associated with low CRP.

Multivariable modelling allowed the estimation of an increase in time to antibiotics of 38 minutes over the 11 years of the study, irrespective of changes in aetiology. These changes are likely multifactorial, but the implication is that additional resources in diagnostics and training are required in order to minimize delays in the treatment of these most invasive infections.

Over 11 years, susceptibility to empirical antibiotic protocols declined. Adults with Gram negative HCA bacteraemia experience delays in appropriate antibiotic therapy,[280] and mortality is increased when initial empirical therapy is inadequate.[282] Death in children is uncommon even in serious infection, and we failed to draw an association between death or likelihood of PICU admission, and inappropriate empirical antibiotics.

3.7.1 Limitations

This was a retrospective, single centre study of culture positive bacteraemia in the paediatric ED. Blood culture yield is affected by the use of prior antibiotics, more so than PCR.[295] Collecting data on prior antibiotics was impractical in this retrospective analysis. Community antibiotic prescribing for children in the UK decreased substantially in the 1990s,[296,297] though there is data to suggest an increase through the course of this study.[298] This would not account for the reduction in pneumococcal bacteraemia, for which there was no temporal trend prior to the introduction of the PCV. An increase in pre-hospital antibiotics may have impacted blood culture yield over time however, particularly if well targeted towards children with bacteraemia.

The analysis failed to account for changes to the population of the hospital catchment area which may have impacted the observed rate of bacteraemia. Instead, ED attendance was incorporated. Over time, there was no change in medical attendances to the ED.

There is as yet no established definition of 'healthcare-associated bacteraemia' in children. The study used a pragmatic definition, based upon the published literature[281]. A number of surrogate markers of frequent exposure to healthcare environments were identified, but it was not possible to collect robust data quantifying how frequently or how recently this exposure may have occurred in individual clinical episodes. Understanding this exposure better, thereby establishing a valid definition of healthcare-associated bacteraemia would help to guide empirical treatment in the ED. The definition was applied consistently across the 11 years of the dataset, and so, as defined, the analysis of temporal trends is robust.

Data on time to antibiotics was missing in approximately 20% of clinical cases, and the study only considered time to empirical therapy, though others have reported time to 'appropriate' therapy.[280] Explanatory variables that may reflect the severity of the clinical presentation (including biomarkers) and that may influence time to antibiotics (such as age) were retrieved from the clinical notes in order to better

understand the variation observed. Other studies have used validated measures of disease severity (such as the Glasgow Meningococcal Septicaemia Prognostic Score) and demonstrated an association with time to antibiotics.[293] A comparable measure of illness severity may help to explain the variation seen, though no such tool is currently in use in all acutely unwell children in the ED.

It has been assumed that the variation in time to antibiotics observed was related to clinical recognition. Time to antibiotics is also used as a measure of crowding in EDs. Some investigators have identified an association between markers of ED crowding and time to antibiotics in young infants,[299] though not consistently.[300] Data on ED crowding was not available. There was no overall increase in numbers presenting to the Alder Hey Children's Hospital ED over the course of the study however, nor was there an association between time of day, day of the week, or time of year – all of which are associated with the volume of ED activity - and time to antibiotics.

3.8 Conclusions

The aetiology of bacteraemia presenting to the Alder Hey children's ED evolved between 2001 and 2011. Increasingly bacteraemia in the children's ED is healthcare-associated. Bacteraemia in the children's ED is now more likely to be resistant to empirical therapy, appears more difficult to recognise and, in the case of HCA bacteraemia is associated with a prolonged length of stay. Prompt and effective antimicrobial treatment of bacteraemia requires improved diagnostic tools alongside continued aetiological surveillance.

Chapter 4: 16S ribosomal RNA PCR (SepsiTest) with sequencing in the evaluation of febrile children presenting to the Emergency Department

4.1 Overview of the chapter

The previous chapter illustrated the changing aetiology of bacteraemia presenting to the children's ED, highlighting the need for prompt pathogen identification to ensure appropriate antimicrobial therapy. In this chapter, I evaluate the use of a broad-range molecular test (SepsiTest) for the diagnosis of bacteraemia in children presenting to the ED.

4.2 Background

4.2.1 Blood culture in children

In clinical practice, the diagnosis of bacteraemia in children is largely dependent upon blood culture. The identification of a causative organism and the ability to evaluate antimicrobial susceptibility ensures that culture methods remain, so far, irreplaceable. The sensitivity of blood culture in children is limited, however, by the volume of blood which is commonly obtained[59,301], and by the use of prior antibiotics[75]. Furthermore, the value of culture is limited by the time taken to achieve a result. Whilst detection time has decreased with automation, median time to positivity in adults with *Streptococcus pneumoniae* is still estimated to be 14h[302]. Following the initial positive identification, a process of phenotypic testing designed to characterise the isolate is undertaken[71]. This process commonly takes days.

The process of phenotypic identification is being supplemented by alternative methods. Matrix assisted laser desorption ionisation-time of flight (MALDI-TOF) is a technique which allows the mass spectrum of organic molecules including bacterial colonies to be analysed, and compared with known profiles. This is a process that can be completed in minutes. Platforms such as MALDI-TOF have established a clinical role in hastening pathogen identification. Shortening the time to identification allows prompt antimicrobial targeting, and has been found to be effective in expediting appropriate antibiotics in the context of an antimicrobial stewardship programme[303]. Such an approach does not address such limitations as the poor sensitivity of blood culture.

4.2.2 Nucleic acid based detection methods

Molecular tests based upon the detection of bacterial genes have the potential to address these limitations of culture methods. The most relevant of the nucleic acid based detection methods is the polymerase chain reaction (PCR). The PCR method amplifies target DNA which is complementary to short DNA primers. These primers are selected to allow the specific amplification of target DNA, such as the DNA sequence specific to an individual pathogen. The method consists of cycles of repeated heating and cooling causing separation of double-stranded DNA into single strands (denaturation) which then become templates for the amplification process. During the cooling step, target DNA present binds the complementary primers (annealing) and with the addition of a DNA polymerase, the primers are extended along the target sequence. This process is then repeated with the resultant DNA now serving as the template, and the quantity of DNA increasing exponentially. In this way, even small quantities of DNA can be amplified within a period of a few hours[304].

4.2.2.1 Pathogen-specific molecular diagnosis

One of the most common applications of the PCR method is in the molecular diagnosis of specific pathogens. Pathogen-specific PCR assays have improved the sensitivity of blood culture for the diagnosis of bacteraemia caused by *Neisseria meningitidis*[76] and *Streptococcus pneumoniae*[259], but are limited by the narrow range of pathogens identified. Following the introduction of effective conjugate vaccines to these important pathogens, their relative contribution to the burden of paediatric bacteraemia has declined[305]. Assays for other important pathogens have been developed, including those capable also of detecting genes encoding antimicrobial resistance such as the *mecA* gene of *Staphylococcus* species[306]. However, the number of potential organisms causing bacteraemia (and other invasive infections) has necessitated a shift towards the simultaneous detection of a range of pathogens.

4.2.2.2 Broad-range molecular tests in the diagnosis of bacteraemia

A number of broad-range molecular tests for the diagnosis of bacteraemia have been developed and evaluated[77] (see Section 1.6.1.2). Such tests employ a variety of approaches. They may be undertaken on positive blood cultures or be culture independent, and use either a multiplex approach, or a 'universal' approach targeting conserved sequences within the bacterial genome. Following bacterial DNA isolation, commonly involving PCR amplification, identification is undertaken using methods such as hybridisation, microarray detection, electrospray ionisation mass spectrometry (ESI MS) or sequencing. Commercially available broad-range assays for the diagnosis of bacteraemia are summarised in Table 21.

Culture independent methods capable of accurately identifying the range of potential bloodstream infections have the potential to both speed accurate pathogen identification, and to improve diagnostic accuracy. Importantly, effective techniques for the extraction of pathogen DNA are essential for this approach to succeed. Bacterial DNA is present in blood in much smaller quantities than host DNA, and whole blood is inhibitory to the PCR reaction[307]. In children from whom only small samples are commonly obtained, these challenges are particularly acute. Various pre-analysis treatment tools have been developed to improve the isolation and selective amplification of pathogen DNA from whole blood[81,308].

SeptiFast (Roche) performs multiplex real-time PCR, is capable of identifying 25 pathogens as well as the *mecA* gene which encodes Methicillin resistance in *Staphylococci,* and has been found to be highly sensitive and specific *in vitro.* The assay is completed in 6 hours[79]. One large study of hospitalised children reported encouraging performance characteristics (sensitivity and specificity 85% and 93.5% respectively) for a composite outcome of bacteraemia defined by SeptiFast, blood culture, and clinical impression[309]. A recent meta-analysis was less encouraging however, and highlighted particularly the limited evidence from children[80].

PLEX-ID (Abbott) is a technique which combines a multiplex PCR step with broad primers for groups of pathogens, and for resistance genes, followed by amplicon detection with ESI MS. Initially evaluated in positive blood cultures, the technique was found to be highly concordant with culture results, and to be substantially quicker[310]. PCR/ESI MS was found to be similarly concordant with MALDI-TOF MS[311]. Practical considerations, including cost, have supported the adoption of the MALDI-TOF platform into clinical practice. Promising recent evaluations of ESI MS in whole blood make this an attractive technology, however[312,313]. The assay proved to be a valuable adjunct to blood culture, though adequate sensitivity was only achieved using 5ml samples of blood. For use in children, this is an important limitation.

4.2.2.3 SepsiTest 16S rRNA PCR with sequencing

SepsiTest (Molzym, Bremen, Germany) is a semi-automated, culture independent diagnostic test based upon PCR amplification of variable regions of the 16S rRNA gene followed by sequence analysis. It has been demonstrated to be a sensitive method for detecting and identifying a broad range of aetiologies of sepsis and infectious endocarditis in adults[83,84]. To date it remains the only commercially approved universal molecular diagnostic for use in whole blood. A pre-analytical stage is used to improve the sensitivity of the SepsiTest assay[82,308]. This 'MolYsis' protocol entails the selective lysis of host cells, followed by the degradation of host DNA by DNAse. Bacterial cells are then lysed and the isolated DNA extracted.

Assay (Manufacturer)	Culture	Technique	Pathogen identification	Range	Reference
FilmArray BCID (BioFire)	Dependent	Multiplex PCR	qPCR by melt curve analysis	19 bacteria, 5 yeasts, *mecA, vanA/B,* KPC	Zheng, 2014[314]
MALDI-TOF MS (Bruker, Biomérieux)	Dependent	MALDI-TOF	MS	Universal	La Scola, 2011[315]
Plex-ID (Abbott)	Dependent	Multiplex PCR/ ESI	MS	Universal	Kaleta, 2011[310]
Prove-it Sepsis (Mobidiag)	Dependent	Multiplex PCR	Hybridisation on microarray	50 organisms, *mecA*	Tissari, 2010[316]
Verigene GP/GN (Nanosphere)	Dependent	Multiplex PCR	Hybridisation on microarray	29 bacteria, 8 yeasts, *mecA, van A/B*	Sullivan, 2013[317]
Magicplex (Seegene)	Independent	Multiplex PCR	qPCR	85 bacteria, 6 fungi, *mecA, vanA/B*	Carrara, 2013[318]
Plex-ID (Abbott)	Independent	Multiplex PCR/ ESI	MS	Universal	Laffler, 2013[312]
SepsiTest (Molzym)	Independent	Broad-range (16S rRNA) PCR	Sequencing	Universal	Wellinghausen, 2009[83]
SeptiFast (Roche)	Independent	Multiplex PCR	qPCR by melt curve analysis	25 organisms, *mec*A	Dark, 2015[80]

Table 21: Commercially available broad-range molecular detection assays for the diagnosis of bacteraemia, including detection of resistance genes *mecA, van A/B,* KPC

4.3 Hypothesis

SepsiTest is a sensitive and specific tool for the diagnosis of bacteraemia in febrile children with clinical evidence of sepsis presenting to the Emergency Department.

4.4 Aims and Objectives

The study set out to evaluate the use of SepsiTest in febrile children in the Alder Hey Children's Hospital NHS Foundation Trust ED. Specifically, the study objectives were to:

I. Describe the performance characteristics of SepsiTest for the diagnosis of bacteraemia in a selected 'high-risk' group of children recruited to the SPICED study
II. Evaluate the use of the biomarkers Procalcitonin, NGAL and Resistin in the prediction of bacteraemia in this group
III. Compare the performance characteristics of SepsiTest in groups of children according to the pre-test likelihood of bacteraemia defined by a combined biomarker risk model

4.5 Methods

4.5.1 Study design

The evaluation of SepsiTest was undertaken as part of the SPICED study, a prospective diagnostic accuracy study of febrile children attending the Alder Hey Children's Hospital Emergency Department (see Methods, 2.2).

4.5.2 Participants

As the risk of bacteraemia in febrile children in the ED is low[6], a sample of children considered to have an increased pre-test probability of bacteraemia was selected for evaluation. This was estimated based upon known clinical indicators of SBI

(temperature, heart rate, respiratory rate, CRP)[10,185] as well as length of hospital stay. Outcome diagnoses at the time of sample selection had not been determined. Together these children constituted a 'high-risk' group. A comparison group of 26 children considered to have a low pre-test probability of bacteraemia was selected from the same diagnostic accuracy study. Risk was again estimated using the clinical criteria alongside CRP. Nearly three quarters of children in the high-risk group met two or more paediatric SIRS criteria [86], as did 43% of those in the low-risk group (73% v 43%, p=0.007). The characteristics of high-risk (n=120) and low-risk (n=26) children are summarised in Table 22.

	High-risk (n=120)	**Low-risk (n=26)**	**p**
Median age / months (IQR)	44 (17-86)	25 (13-47)	0.06
Male / % (95% CI)	60 (51-69)	54 (34-73)	0.56
Significant comorbidity / % (95% CI)	34 (26-43)	19 (7-40)	0.14
Clinical variables/Median (IQR)			
Temperature	38.1 (37.2-39.1)	37.7 37.0-38.6)	0.20
Heart rate	144 (119-170)	132 (120-142)	0.16
Respiratory rate	30 (24-38)	28 (24-32)	0.31
Biomarkers/Median (IQR)			
CRP / mg/dl	93 (65-137)	6 (0-12)	<0.001
WCC / x10^9/l	14.3 (10.7-18.4)	8.99 (6.08-11.2)	<0.001
PCT / ng/ml	0.97 (0.40-4.22)	0.19 (0.08-0.34)	<0.001
≥2 SIRS criteria / %	73.1	42.9	0.007
Median LOS / days	3	0	<0.001
SBI / % (95% CI)	43 (34-53)	0	<0.001

Table 22: Clinical features of children included in the evaluation of broad-range 16S rRNA with sequencing study. IQR: interquartile range; LOS: length of hospital stay.

4.5.3 Outcome diagnosis

Outcome diagnoses for children recruited to the diagnostic accuracy study were independently determined by a Paediatric Infectious Disease consultant and a Paediatric Infectious Disease registrar using pre-defined composite reference standards. In the case of disagreement, a second Paediatric Infectious Disease consultant considered the evidence and consensus was reached. This approach reflects the lack of a 'gold' reference standard for SBIs in children.

Bacteraemia was defined as:

- the identification of a relevant pathogen by any method (culture, pathogen-specific PCR or SepsiTest)
- the identification of a possible pathogen in two blood cultures, or corroborated by two different methods
- the identification of a possible pathogen in culture or SepsiTest, with supportive clinical evidence of bacteraemia. Clinical evidence of bacteraemia was considered to be signs of sepsis on presentation, alongside elevated markers of infection (in this case, WCC and CRP), prolonged administration of intravenous antibiotics, and prolonged hospital stay despite negative culture results
- clinical evidence of bacteraemia (as described) with supportive microbiological evidence of a relevant pathogen (for example, an acute serological response to *Streptococcus pneumoniae* in a child presenting with signs of Streptococcal septicaemia).

The details of this evaluation are summarised in Table 23.

Culture	Specific PCR	SepsiTest	Clinical signs	Supportive microbiology	Outcome
Pathogen	±	±	±	±	Bacteraemia
±	Pathogen	±	±	±	Bacteraemia
±	±	Pathogen	±	±	Bacteraemia
Possible pathogen:					
Single isolation	-ve	Same organism	±	±	Bacteraemia
	-ve	-ve	+ve	±	Bacteraemia
	-ve	-ve	-ve	±	No bacteraemia
Repeated isolation	-ve	±	±	±	Bacteraemia
Negative	-ve	Possible pathogen	+ve	±	Bacteraemia
			-ve	±	No bacteraemia
Negative	-ve	Negative	+ve	+ve	Bacteraemia
			+ve	-ve	No bacteraemia
			-ve	±	No bacteraemia

Table 23: Outcome diagnosis of bacteraemia according to microbiological and clinical evidence

4.5.4 Conventional microbiology evaluation

Blood culture bottles (BacT/ALERT PF, bioMeriéux, Marcy-l'Etoile, France) were inoculated with 1ml blood collected from a venepuncture and incubated for up to five days. Additional tests were undertaken according to routine clinical practice and at the discretion of the clinical team. This included the use of pathogen-specific PCR assays for *Neisseria meningitidis* and *Streptococcus pneumoniae*. Pathogen-specific PCRs were performed at the Meningococcal Reference Unit in Manchester, UK [259].

4.5.5 SepsiTest molecular analysis

Blood was drawn into EDTA collection tubes from which 1ml was withdrawn and stored at -80°C in UMD-Tubes (Molzym, Germany) until analysis. Frozen samples were shipped to Bremen on dry ice, to be analysed in the Molzym laboratory. SepsiTest analysis was undertaken blinded to outcome diagnosis. Thawed samples

underwent a process of pathogen amplification using a chaotropic buffer containing guanidine hydrochloride to selectively lyse host cells. Host DNA was then degraded using DNAse, and intact bacterial cells recovered by centrifugation. The procedure was continued automatically with the lysis of microorganisms, extraction of the sample and isolation of DNA by a magnetic bead-based protocol. The eluate was subsequently included in Real-Time PCR targeting conserved sites for the amplification of variable regions of the 16S rRNA (V3/V4) and 18S rRNA (V9) genes respectively. Amplifications were performed in a DNA Engine Opticon cycler (Bio-Rad, Hercules, USA) following the recommended programme in the SepsiTest SelectNA™ manual. Following a positive reaction, amplicons were sequenced using supplied sequencing primers. Editing by visual inspection was directed to the correction of single bases if unrecorded because of signalling below the set threshold. Strains were identified using SepsiTest-BLAST (http://www.sepsitest-blast.net). At sequence identities >97 and ≥99% genus and species names were filed, respectively. Mixed sequences (i.e., chromatograms showing overlapping sequences of two or more different sequences) were analysed using the RipSeq mixed programme (Isentio, Bergen, Norway). This tool uses an algorithm for the separation of mixed sequences which are then identified by Blast analysis. Cases positive in the 16S PCR assay and with mixed sequences that could not be resolved were assigned positive for bacteria.

4.5.6 Biomarker analysis

Blood (0.5-1.0ml) taken at the same time as routine clinical tests including blood culture, was collected into Lithium Heparin and centrifuged at 13,000 rpm for 5 minutes. Plasma was removed and stored in Sarstedt 0.5ml microtubes at -80°C. Sample freezing occurred within 1 hour. Prior to analysis the samples were thawed, vortex mixed and centrifuged again to remove bubbles and particulate matter. Procalcitonin (PCT) analysis was undertaken on the B.R.A.H.M.S. Kryptor platform (Thermofisher scientific, e Hennigsdorf). Samples were analysed according to manufacturer's instructions, in batches of 50. Quality control samples were analysed at the beginning and end of a run. Neutrophil Gelatinase-associated Lipocalin (NGAL, R&D systems, Minneapolis) and Resistin (Assaypro, Missouri) were

analysed using validated commercial ELISA according to the manufacturer's instructions. Intra and inter-plate variability were monitored.

4.5.7 Statistical methods

Statistical analysis was undertaken in R, version 3.0.3[263]. Continuous data were described by median and interquartile range, and comparison between groups performed using the Mann-Whitney U-test. Categorical variables were expressed as percentages with 95% confidence intervals (95% CI). Groups were compared by means of a χ^2 test. In order to compare the performance characteristics of blood culture and SepsiTest, and the proportion of VGS bacteraemia between the two groups, a Monte Carlo simulation was used to check the accuracy of the asymptotic approximation of the null sampling distribution.

For the diagnosis of bacteraemia, performance characteristics of the biomarkers individually, and in combination, were evaluated using the epiR package in R[319]. Receiver operating characteristic (ROC) curves were plotted in the pROC package[320]. The biomarker models were used to estimate the probability of bacteraemia from which multiple risk thresholds were derived. Using these thresholds, we evaluated the performance characteristics of SepsiTest in isolation, and SepsiTest in combination with blood culture. In this scenario, all children were considered to have undergone testing with blood culture, while the use of SepsiTest was limited to children above each stated risk threshold. The impact of this testing strategy on the whole group was estimated.

4.5.8 Ethics approval

Children were recruited to the study following written, informed consent. Ethical approval was granted by the Greater Manchester West Research Ethics Committee (10/H1014/53) and site approval granted by the Alder Hey Children's NHS Foundation Trust Research and Development department.

4.6 Results

Sixteen children (11%, 95% CI 6 to 17%) were diagnosed with bacteraemia. This was confirmed microbiologically (by any method) in thirteen cases. Two had concurrent meningitis (both *Streptococcus pneumoniae*).

4.6.1 Performance characteristics of blood culture and SepsiTest

The performance characteristics of both SepsiTest and blood culture are detailed in Table 24. Though the sensitivity of two tests was not significantly different, the high positivity rate of SepsiTest translated into a significantly less specific test (68% v. 95%, p=0.02). The addition of SepsiTest to blood culture evaluation improved the sensitivity of diagnosis from 63% to 75% at the expense of specificity (95% to 66%).

16S PCR with sequencing (SepsiTest)	**Bacteraemia +**	**Bacteraemia -**	**Total**
Test +	9	41	50
Test -	7	89	96
Sensitivity (95% CI)	56% (30 to 80%)		
Specificity (95% CI)	68% (60 to 76%)		
PLR	1.78 (1.08 to 2.94)		
NLR	0.64 (0.36 to 1.13)		
Blood culture	**Bacteraemia +**	**Bacteraemia -**	**Total**
Test +	10	6	16
Test -	6	124	130
Sensitivity (95% CI)	63% (35 to 85%)		
Specificity (95% CI)	95% (90 to 98%)		
PLR	13.5 (5.68 to 32.3)		
NLR	0.39 (0.21 to 0.74)		
SepsiTest and blood culture combined	**Bacteraemia +**	**Bacteraemia -**	**Total**
Test +	12	44	56
Test -	4	86	90
Sensitivity (95% CI)	75% (48 to 93%)		
Specificity (95% CI)	66% (57 to 74%)		
PLR	4.82 (1.64 to 14.2)		
NLR	0.82 (0.71 to 0.94)		

Table 24: Performance characteristics of blood culture and SepsiTest 16S rRNA PCR with sequencing for the diagnosis of bacteraemia in the children's Emergency Department.

The overall positivity rate of SepsiTest in febrile children in the ED was 50/146 (34%, 95% CI 27 to 43%). A further five positive tests were discounted as environmental contaminants. Sixteen positive blood cultures were obtained (11%, 7 to 17%) of which 6 were considered contaminants. Blood culture and SepsiTest results were concordant in 8/16 cases of bacteraemia.

Both methods identified the same 2 of 5 cases of meningococcal septicaemia, the remainder of which were confirmed by specific PCR. SepsiTest identified a *Streptococcus dysgalactiae* in the context of a child with chickenpox, and negative blood cultures. It further identified a Group A Streptococcus and *Klebsiella pneumoniae* in a mixed central venous line infection which cultured only *K. pneumoniae*, and a *Streptococcus mitis* in a culture-negative presumed septicaemia.

There were significantly more positive SepsiTest identifications than positive blood cultures. Of particular note, Viridans Group Streptococci (VGS) were identified by SepsiTest in 17 children. All of these identifications occurred in the high-risk group (17/120 v 0/26, p=0.06). The clinical characteristics of children with VGS bacteraemia are described in Table 25. Additional positive identifications of bloodstream infections caused by *Gemella haemolysans* (in a child with pneumonia), *Moraxella osloensis* (pneumonia) and *Enterococcus* spp. (orbital cellulitis) were made by SepsiTest.

Age / months	Sex	Clinical diagnosis	CRP	LOS	Identification of VGS	Additional microbiology (source)
58	M	URTI	67.1	0	Streptococcus salivarius	Group A Streptococcus (throat swab)
16	F	URTI	56.6	4	Streptococcus sanguinis	
19	M	URTI	125.2	3	Streptococcus mitis group	
59	M	URTI	91.5	0	Streptococcus salivarius	
125	F	Bacteraemia	172.2	3	Streptococcus mitis group	
12	M	URTI	52.5	1	Streptococcus mitis group	
73	M	Peri-orbital cellulitis	61.9	2	Streptococcus mitis group	
66	M	Dental abscess	265.5	2	Streptococcus mitis group	
85	M	Lymphadenitis	153	0	Streptococcus thermophilus	
40	M	LRTI	134.6	1	Streptococcus mitis group	
1	M	LRTI	71	3	Streptococcus cristatus	
6	M	LRTI	67.4	11	Streptococcus peroris	Respiratory syncytial virus (nasopharyngeal aspirate)
15	M	LRTI	73.2	1	Streptococcus salivarius	
21	F	Meningococcal sepsis	364.5	7	Streptococcus salivarius	Neisseria meningitidis (blood)
31	M	Cellulitis	85.8	2	Streptococcus peroris	
64	M	Gastroenteritis	63.4	1	Streptococcus parasanguinis	
87	F	Pyelonephritis	263.1	5	Streptococcus thermophilus	Escherichia Coli (urine)

Table 25: Clinical characteristics of febrile children with VGS bacteraemia. URTI: upper respiratory tract infection; LRTI: lower respiratory tract infection.

4.6.2 Risk stratification using the biomarkers PCT, NGAL and Resistin

The biomarkers PCT, NGAL and Resistin all demonstrated reasonable discrimination between children with bacteraemia and those without (Area under the ROC curve 0.80, 0.81, and 0.71 respectively, Figure 14).

The combination of the three biomarkers improved the fit of the model to the data ($p<0.001$), though this did not translate into an improvement in the AUC (0.80, and 0.79 respectively, $p=0.8$; Figure 15). Performance characteristics for the combined biomarker model at various risk thresholds are recorded in Table 26. At a risk threshold of 10%, the positive likelihood ratio indicated a significantly increased likelihood of bacteraemia (PLR 3.32, 95%CI 1.75 to 6.30).

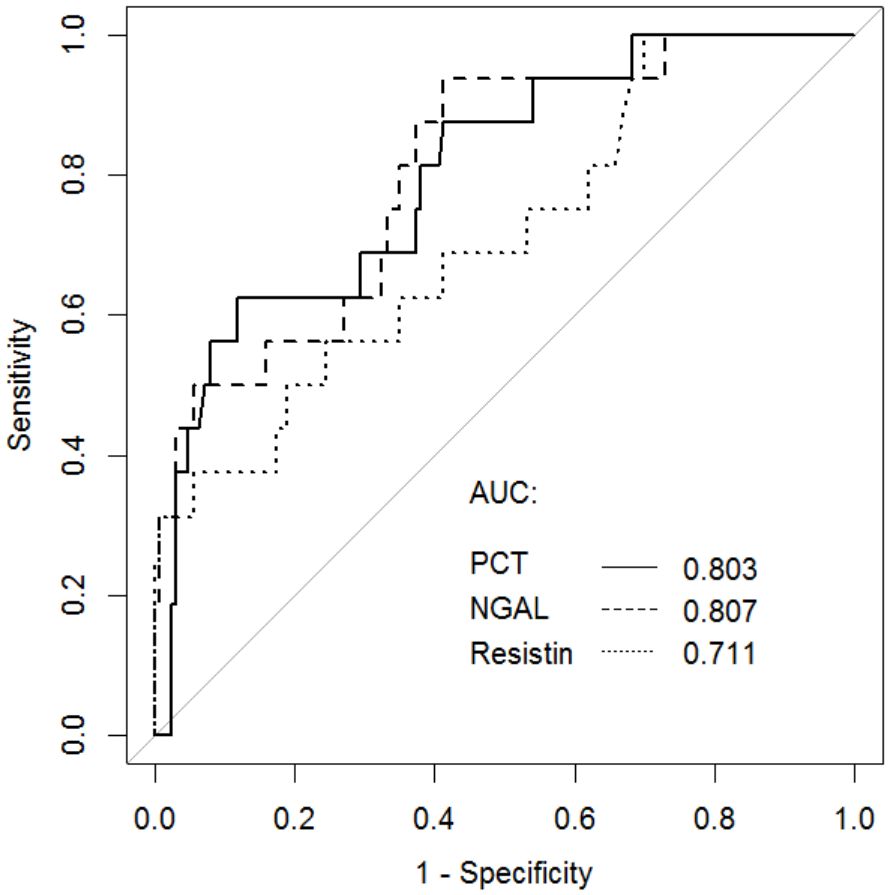


Figure 14: ROC curves for PCT, NGAL and Resistin in the diagnosis of bacteraemia

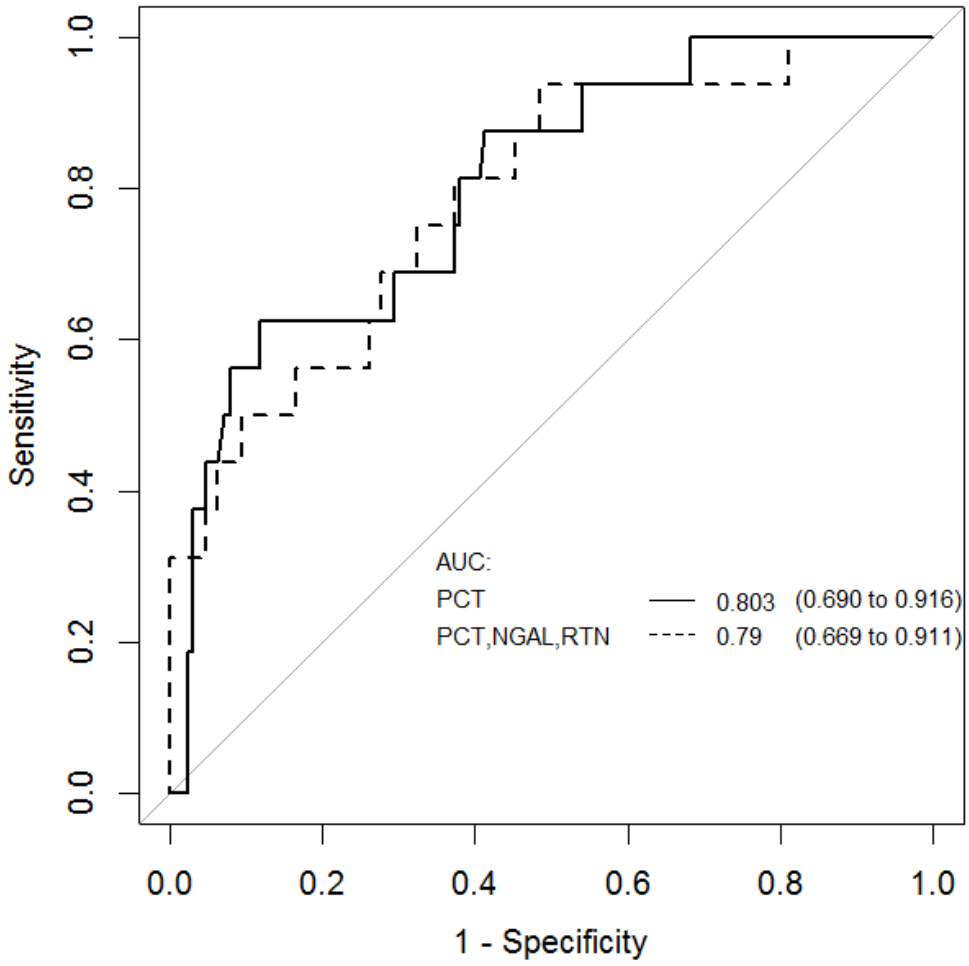


Figure 15: ROC curves for PCT alone, and in combination with NGAL and Resistin (RTN) for the diagnosis of bacteraemia

4.6.3 Use of SepsiTest in risk groups defined by the combined biomarker model

The biomarker model was used to define risk groups in which to estimate the performance of SepsiTest. Table 27 details the performance characteristics of SepsiTest in those children above each listed risk threshold, for the diagnosis of bacteraemia. Limiting the use of SepsiTest to groups at increasing risk of bacteraemia reduced the number of false positive identifications but was insufficiently sensitive to confidently rule out bacteraemia in this selected group of children.

Risk	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	PLR (95% CI)	NLR (95% CI)
5%	1 (0.71 - 1.00)	0.09 (0.04 - 0.15)	0.12 (0.07 - 0.19)	1 (0.62 - 1)	1.10(1.04 - 1.16)	NaN (NaN)
10%	0.5 (0.25 -0.75)	0.85(0.77 - 0.91)	0.3 (0.14 - 0.50)	0.93 (0.87 - 0.97)	3.32(1.75 - 6.30)	0.59 (0.36 - 0.97)
20%	0.38 (0.15 -0.65)	0.95(0.90 - 0.98)	0.5 (0.21 - 0.79)	0.92 (0.86 - 0.96)	7.88(2.88 - 21.5)	0.66 (0.45 - 0.96)
30%	0.31 (0.11 -0.59)	0.99(0.96 - 1.00)	0.83 (0.36 - 1.00)	0.92 (0.86 - 0.96)	39.40(4.90 - 316)	0.69 (0.50 - 0.96)

Table 26: Performance characteristics of the combined biomarker model for the diagnosis of bacteraemia at various risk thresholds

Risk	n	Bacteraemia (%)	SepsiTest				Sensitivity (95% CI)	Specificity (95% CI)	PLR (95% CI)	NLR (95% CI)
			TP	FP	FN	TN				
All	146	16 (11)	9	41	7	89	56 (30 to 80)	68 (60 to 76)	1.78 (1.08 to 2.94)	0.64 (0.36 to 1.13)
5%	131	16 (12)	9	35	7	80	56 (30 to 80)	70 (60 to 78)	1.85 (1.11 to 3.09)	0.63 (0.36 to 1.11)
10%	27	8 (30)	5	5	3	14	63 (24 to 91)	74 (49 to 91)	2.38 (0.94 to 5.98)	0.51 (0.20 to 1.30)
20%	12	6 (50)	4	2	2	4	67 (22 to 96)	67 (22 to 96)	2.00 (0.56 to 7.09)	0.50 (0.14 to 1.77)
30%	6	5 (83)	4	0	1	1	80 (28 to 99)	100 (1 to 100)	Inf (-Inf to Inf)	0.20 (0.03 to 1.15)

Table 27: Performance characteristics of SepsiTest for the diagnosis of bacteraemia applied to children above the listed risk thresholds, defined by the biomarker model. TP: True positive, FP: False positive, FN, False negative, TN: True negative.

Risk /%	SepsiTest						Combined SepsiTest and Blood culture (n=146)							
	n	Bacteraemia (%)	TP	FP	FN	TN	TP	FP	FN	TN	Sensitivity (95% CI)	Specificity (95% CI)	PLR (95% CI)	NLR (95% CI)
All	146	16 (11)	9	41	7	89	12	44	4	86	75 (48 to 93)	66 (57 to 74)	2.22 (1.53 to 3.21)	0.38 (0.16 to 0.89)
>5	131	16 (12)	9	35	7	80	12	39	4	91	75 (48 to 93)	70 (61 to 78)	2.50 (1.70 to 3.68)	0.36 (0.15 to 0.84)
>10	27	8 (30)	5	5	3	14	11	11	5	119	69 (41 to 89)	92 (85 to 96)	8.13 (4.22 to 15.6)	0.34 (0.16 to 0.71)
>20	12	6 (50)	4	2	2	4	11	8	5	122	69 (41 to 89)	94 (88 to 97)	11.2 (5.29 to 23.6)	0.33 (0.16 to 0.69)
>30	6	5 (83)	4	0	1	1	11	6	5	124	69 (41 to 89)	95 (90 to 98)	14.9 (6.38 to 34.8)	0.33 (0.16 to 0.68)

Table 28: Performance characteristics of a combination of SepsiTest used in children above the listed thresholds defined by the biomarker model, along with blood culture in all children. TP: True positive, FP: False positive, FN, False negative, TN: True negative.

Of more interest is how the application of SepsiTest at various risk thresholds impacted the diagnosis of bacteraemia in the whole group (Table 28). This assumes that SepsiTest is performed only in children above each listed threshold, but supplemented by blood culture in all. Limiting the use of SepsiTest only to the most high-risk children reduces the number of false positive identifications, and results in improvements in specificity and PLRs when compared with the use of SepsiTest in all children.

As an illustration, when SepsiTest is applied to the whole group, 146 additional tests are performed. For this diagnostic investment, two additional cases of bacteraemia are identified in addition to those identified by culture. Thirty-eight additional false positives result. By restricting SepsiTest to children with a biomarker-predicted risk >10%, 27 additional SepsiTest investigations are performed, identifying a single case of bacteraemia in addition to those identified by blood culture. In this case, there are only two additional false positives to consider. The PLR of this diagnostic approach is 8.13 (95% CI 4.22 to 15.6).

4.7 Discussion

In febrile children in the ED with a significant pre-test probability of sepsis, SepsiTest broad-range 16S-rRNA PCR with sequencing augmented the use of blood culture in the diagnosis of bacteraemia. In this first evaluation of SepsiTest in children using samples of approximately 1ml of blood, the performance characteristics of SepsiTest and blood culture were broadly comparable. The use of the two tests in combination yielded better sensitivity than either test in isolation.

Both blood culture and broad-range PCR failed to identify significant pathogens. Of five cases of meningococcal septicaemia, SepsiTest and blood culture both identified the same two cases, but failed to identify a further three confirmed by meningococcal-specific PCR. It is possible that the concentration of pathogen DNA in these cases was below the limit of detection [83,84]. Alternatively, while pathogen-specific PCR detects DNA from both viable and killed organisms, the SepsiTest pre-

analytical stage degrades free DNA from killed and lysed organisms which may explain the negative results[321]. For diagnostic purposes, this is an important limitation.

The use of pathogen-specific PCR is well established for the purpose of improving yield in the diagnosis of MCD. Highly sensitive PCR assays for MCD have demonstrated superior performance characteristics to blood culture[295] and are widely used in paediatric practice in the UK. The molecular diagnosis of invasive pneumococcal disease (IPD) using PCR has proven more challenging, but recent PCR assays targeting the autolysin gene of *S.pneumoniae* have been demonstrated to be more sensitive than blood culture in both adults and children, and in contrast to those targeting the pneumolysin gene, highly specific[322-324]. The limitations of pathogen-specific diagnostics are, of course, that while the test may be highly sensitive for a suspected pathogen, its sensitivity for the diagnosis of bacteraemia overall is poor – a negative test only rules out a single pathogen. Broad-range methods, such as 16S rRNA PCR, offer the potential of identifying all possible causative organisms, but to date remain insufficiently sensitive to reliably rule out bacteraemia[83].

The yield from the use of SepsiTest in all children in our pre-selected sample is relatively small. The test is insufficiently sensitive for a negative test to rule out bacteraemia. The test benefits can be summarised as the identification of two additional cases of bacteraemia and the potential of reduced time to identification in other concordant cases. Such benefits need to be measured against the costs involved. Furthermore, the frequent positive identification of organisms of uncertain significance may have unintended consequences including unnecessary antimicrobial therapy and prolonged hospital admission. The use of risk stratification may have a role in optimising the use of the test, providing supplementary diagnostic information to blood culture in those most likely to have bacteraemia in a shortened time frame, while limiting the number of false positive identifications.

There is no gold standard for the diagnosis of bacteraemia, which makes diagnostic accuracy studies difficult to interpret. The performance characteristics of the SepsiTest have been estimated against a pre-defined outcome composite reference diagnosis of bacteraemia. The approach risks incorporation bias however, as the identification of a 'true pathogen' on SepsiTest was incorporated into the definition of bacteraemia. By contrast plausible organisms not commonly associated with the diagnosis of bacteraemia were only considered 'true' if associated with corroborative findings as interpreted by the investigators. This is an accepted approach to the problem of imperfect reference standards[64], and more appropriate than a direct comparison with blood culture.

Alternative approaches (discussed in Section 1.5.2) might include, for example, latent class analysis where the results of the SepsiTest assay were considered as one of a number of predictor variables for the true unknown 'latent' state. Such an approach may yield more accurate estimates of diagnostic performance, but the clinical benefit would be unclear. SepsiTest, for example, identified a number of plausible pathogens in children who received an outcome diagnosis other than bacteraemia. *Gemella haemolysans* and *Moraxella osloensis* were both identified by SepsiTest in the bloodstream of children with significant respiratory infections. Though typically opportunistic, both have been described in children with invasive infections [325,326]. It is possible that these children, alongside a child with post-septal cellulitis in which SepsiTest identified Enterococcus were wrongly considered not to have bacteraemia. In order to demonstrate the value of these SepsiTest identifications, it would be necessary to demonstrate that the disease state defined by the latent class model was of clinical interest. That is, are these children of similar severity to those with blood culture-positive bacteraemia, do they require the same treatment, and are their outcomes the same?

The finding of VGS bacteraemia in this selected high-risk group of febrile children is interesting. The fact that no VGS bacteraemia was identified in a low-risk comparison group of febrile children sampled in the same way argues against this being a problem relating to sample contamination. VGS are abundant oral commensals. By the age of one year, *Streptococcus mitis*, *S. parasanguinis* and *S.*

oralis predominate in the oral flora[327]. Whilst typically benign organisms of the upper respiratory tract, invasive infections of the immunocompromised host are well recognised [328,329]. VGS are also a common cause of infective endocarditis in immunocompetent children and adults[330], and one of the most commonly identified bloodstream infections in the UK[331]. In one study of community-acquired bacteraemia in the UK, 12% of all paediatric cultures were VGS (13/106, of which 7 were of 'probable' clinical significance) although this is at odds with our retrospective analysis of bacteraemia in the ED (6/575, 1%; Chapter 3). Most of these children presented with acute respiratory infections, as in our study[332]. This association has also been made in children with fever and neutropenia[333]. It is unclear why we identified VGS bacteraemia with such frequency in culture negative samples. Conceivably, intracellular VGS are released into the blood sample during the pre-analytical lysis of host cells. This finding deserves further exploration.

The mechanisms by which VGS cause invasive infection is of increasing interest[334]. *Streptococcus mitis* binds platelets via phage proteins PbIA and PbIB, and the loss of expression of these proteins results in decreased virulence in animal models of endocarditis[335]. Similarly, it is hypothesised that *S. parasanguinis* is implicated in endocarditis by its ability to bind platelets[336]. *Streptococcus mitis* shares significant homology with the highly pathogenic *S. pneumoniae*, and the recently completed genome of *S. mitis*[337] and *S. oralis*[338] isolates have highlighted the presence of genes analogous to virulence genes present in *S. pneumoniae*.

Whether VGS in the bloodstream of this group of acutely unwell children contribute to the clinical picture, mediating the inflammatory response and conferring morbidity is unclear. The finding may indeed be a response to a profound inflammatory illness during which alterations to mucosal immunity allow the invasion of oropharyngeal organisms into the bloodstream. VGS have the potential to cause significant invasive infection and the possibility that a significant proportion of immunocompetent children with acute febrile illnesses experience a VGS bacteraemia deserves greater examination. VGS bloodstream isolates are commonly resistant to antibiotics[331], and have the potential to transfer

chromosomal DNA to *S. pneumoniae* resulting in high-level resistance[339]. This is a clinical scenario with significant implications.

4.7.1 Comparison with other studies

A number of broad-range molecular approaches to the diagnosis of bacteraemia have been developed and evaluated, mostly in adults [77,340]. Fewer studies have been undertaken in children. A large study of the use of a multiplex PCR (SeptiFast, Roche, Germany) in children supported its use as an adjunct to blood culture, and suggested a shortened time to pathogen identification [309]. A number of small studies have investigated the use of 16S rRNA PCR in neonates [341-343], with variable results. This is the first study of this approach in the children's ED.

The value of SepsiTest in the children's ED is in expediting pathogen identification in those at risk, that is, in ruling in bacteraemia and guiding appropriate treatment. In our high-risk sample, the use of SepsiTest in 120 children yielded additional information to blood culture in only two children. This may be considered clinically worthwhile, particularly if these and other concordant results are available to clinicians more quickly than culture, but further stratifying risk in this setting may be appropriate. This approach was taken in a study of adults in ED, though in this setting, SepsiTest performed poorly in comparison to blood culture [344]. In our study, applying a risk threshold of 10% to a biomarker based model limited the use of the test to 31 children, in whom 7 had bacteraemia, five of which were identified by SepsiTest. One *Streptococcus dysgalactiae* bacteraemia was not identified by either culture, or pathogen-specific PCR. The test would be insufficiently sensitive in this group to rule out bacteraemia and stop treatment, but may have value in guiding appropriate antimicrobial therapy if the results are available to clinicians prior to blood culture. At a time when the aetiology of bacteraemia in the children's ED is evolving, this is important [305].

The broad-range identification of pathogens in small volumes of blood requires a process of host DNA removal and pathogen amplification. The pre-analytical

MolYsis protocol has been demonstrated to reduce host DNA, but at the expense of reduced bacterial load[345]. Concern has been expressed that the protocol may particularly affect Gram negative organisms with a relatively more fragile cell wall, and that this approach would not be successful for the diagnosis of Gram negative organisms[346]. We have demonstrated the successful diagnosis of Gram negative septicaemia in small samples obtained from children, though the lack of sensitivity for *N.meningitidis* is of concern.

4.7.2 Limitations

For pragmatic reasons, our analysis was undertaken in a sample of children considered to have an increased pre-test probability of bacteraemia. Given that the likelihood of bacteraemia in febrile children in ED is estimated to be 1 in 250[6], the yield of the test in all children in this setting would have made the study impractical. This pragmatic approach to study recruitment yielded in effect a 'case-control' design in which the performance of SepsiTest was compared in children considered likely to have the target condition (bacteraemia) versus those considered unlikely. Inclusion in the study was non-consecutive. Both are characteristics of diagnostic studies which are recognised to overstate diagnostic accuracy. The most striking example of this occurs in studies which recruit severe cases and healthy controls. Studies of this kind have been shown to yield Diagnostic Odds Ratios for index tests of approximately 5 times that of more appropriately designed studies[56,347]. We avoided this particular bias by studying febrile children in the ED, but recognise the bias in the selection strategy.

Further stratification of the sample using a biomarker based risk model suggests that it may be possible to target the use of the test in the highest risk children. However, other than asserting that this was a selected 'high-risk' group of children, it is not possible to generalise to which children the test may be of value. We advocate for future studies to evaluate the use of the test in high-risk children stratified by externally validated risk prediction models appropriate to the children's ED[6,96].

Molecular analysis was not undertaken in real time, so we are unable to comment on the logistics of the method. One attraction of the molecular approach to diagnosis is in reducing time to pathogen identification, and expediting appropriate antimicrobial therapy. The SepsiTest method evaluated is a semi-automated method, which undertakes the pre-analytical phase and PCR within 4h, requiring 75 minutes hands on laboratory time. Sequencing is estimated to require a further 4h and requires the skills to interpret and possibly edit the sequence output. Whilst the treatment of bacteraemia in the ED would remain largely empirical, and based upon local epidemiology, SepsiTest and other broad-range molecular platforms may have a role in optimising rational and effective antimicrobial therapy by expediting pathogen identification.

As the aetiology of invasive infections in children evolves, it is increasingly important to make a rapid aetiological diagnosis which current methods fail to achieve. In order for clinical improvements to be realised, broad-range molecular tests such as SepsiTest will need to be incorporated into models of care integrating laboratory services with appropriate clinical expertise. Improvements in antimicrobial stewardship have been achieved by combining new diagnostic technologies (such as MALDI-TOF MS[303] and Verigene BC-GP[348]) with clear multidisciplinary strategies for reporting timely results, and communicating clinical decisions. It is in this context that it is possible to suggest the use of SepsiTest in high-risk children as an adjunct to established methods.

4.8 Conclusions

SepsiTest broad-range PCR and sequencing augmented the use of culture in the diagnosis of bacteraemia in children in the ED. It is insufficiently sensitive to rule out bacteraemia, but in expediting pathogen identification, it may have a role in optimising and rationalising empirical antimicrobial therapy in high-risk children. SepsiTest is possible in small volume paediatric samples. Further, it identified bloodstream infections caused by organisms with the potential to confer significant morbidity including VGS. Risk stratification using a combination of biomarkers may identify high-risk children in whom the test has the greatest value. This is a tool with potential value to support the culture-based diagnosis of bacteraemia in febrile children.

Chapter 5: The derivation and validation of a multivariable risk prediction model for the diagnosis of Serious Bacterial Infection in the children's Emergency Department

5.1. Overview of the chapter

In this chapter, the focus will shift from bacteraemia, an important but uncommon SBI in the children's ED, to the diagnosis of all SBI in the ED. I will present the results of a large prospective diagnostic accuracy study of febrile children presenting to the ED (the SPICED study) with a particular focus on the derivation and internal validation of a multivariable clinical risk prediction model for the diagnosis of 'pneumonia' and 'other SBIs'.

5.2. Background

5.2.1. Serious bacterial infections in the children's Emergency Department

Acute infections make up a large proportion of all attendances to the children's ED[3]. Differentiating SBI from self-limiting infection in this setting is challenging. The likelihood of SBI in febrile children under 5 years presenting to the ED is approximately 7%, the majority of which are lower respiratory or urinary tract infections[6].

Prompt recognition of SBI in children is essential for effective management. Children with meningococcal disease are frequently missed at initial presentation[45], and delays in the recognition and management of severe sepsis increase mortality[15,16]. Though rates of invasive infections attributable to *Neisseria meningitidis, Haemophilus influenzae* and *Streptococcus pneumoniae* have declined in recent decades following the introduction of conjugate vaccines[12,20,287,349], SBI continues to account for a substantial proportion of childhood morbidity and mortality[37].

The challenge of differentiating SBI from self-limiting infection is not limited to a failure of recognition of serious infection. Difficulty in ruling out SBI also has serious implications. Despite declining rates of invasive infections presenting to the ED, the likelihood of a child being admitted to hospital has increased over the last decade[49]. The greatest increase is in young children with uncomplicated short stay admissions

for acute infections, in whom the rate of admission approximately doubled between 1999 and 2010[22]. Many of these admissions are for 'primary-care sensitive' conditions, that is, conditions which are considered appropriate to treat in the community.

Failure to rule out SBI also limits rational antimicrobial use. Antimicrobial consumption is associated with an increasing rate of antimicrobial resistance (AMR)[350], and global efforts to mitigate the impact of AMR include a focus on antimicrobial stewardship[351]. Improving the ability of clinicians to confidently rule out SBI in the ED could make a valuable contribution to improving rational decision-making regarding both admission to hospital, and antimicrobial use.

5.2.2. The diagnosis of SBI in the children's ED

The assessment of the febrile child includes the evaluation of multiple clinical variables alongside the use of adjunctive tests. Numerous studies have reported the performance characteristics of these clinical[10] and laboratory[185] variables in the diagnosis of paediatric SBI in the ED. Presently in the UK, the approach to the febrile child is guided by the NICE "Feverish illness in children" guideline[184]. This is a consensus guideline designed for use in children under 5 years, and makes use of a 'traffic light' system to categorise risk. Recently, the guideline has undergone evaluation and been found to have limited capacity to discriminate between serious and self-limiting infections[249,250,352].

Recognising the limited ability of individual clinical signs, or biomarker tests to differentiate between SBI and self-limiting infections, numerous authors have sought to combine clinical and biomarker variables in clinical prediction rules or models. Two large prospective studies derived and internally validated risk prediction models comprising clinical variables[6,9], and another demonstrated the value of the addition of the biomarker C-Reactive Protein (CRP) to a clinical model[96]. An alternative approach has been to build a 'Lab score' made up of CRP, Procalcitonin (PCT) and urinalysis[7,253]. A previous study from the University of Liverpool, investigated Malawian children with pneumonia and meningitis, and demonstrated that a combination of the biomarkers PCT, Resistin, and Neutrophil

Gelatinase-associated Lipocalin (NGAL) improved diagnostic accuracy over any one individual marker[221].

Despite the demonstration of robust discrimination, studies of diagnostic tests in childhood SBI have so far failed to significantly impact clinical practice. A recent Health Technology Assessment review highlighted methodological weaknesses limiting the external validity of the findings of diagnostic accuracy studies. One recommendation was to avoid restrictive inclusion criteria, such as age, temperature, or clinical syndrome in order that study findings might be more appropriately generalised to other populations[64].

5.3. The SPICED study

The SPICED study was a prospective diagnostic accuracy study undertaken in febrile children (up to 16 years of age) presenting to the ED.

5.4. Hypothesis

The combination of clinical and biomarker variables in a risk prediction model discriminates between SBI and no SBI, and between pneumonia, 'other SBIs' and no SBI.

5.5. Aims and objectives

The study sought to evaluate the use of clinical and biomarker variables for the diagnosis of SBI in febrile children of all ages presenting to the ED.

Specifically, the study objectives were:

I. To derive and validate a multivariable model including established clinical and biomarker variables alongside the biomarkers PCT, NGAL and Resistin for the diagnosis of SBI in this population of children.
II. To derive and validate a multivariable risk prediction model to discriminate between pneumonia, 'other SBIs' and no SBI.
III. To determine the value of salivary PCT as a marker of SBI in this population of children

5.6. Methods

This was a prospective study of the diagnostic accuracy of clinical and biomarker variables for the diagnosis of SBI in children presenting to the Alder Hey Children's Hospital ED. The study methods are detailed in chapter 2. The study is reported in line with the published Standards for Reporting of Diagnostic accuracy studies (STARD)[353].

5.6.1. Ethics approval

Ethics approval for the study was granted by the Greater Manchester West Research Ethics Committee (10/H1014/53), and site approval granted by the Alder Hey Children's NHS Foundation Trust Research and Development department.

5.6.2. Saliva as a non-invasive sample for the detection of PCT

There is no validated assay for the measurement of PCT in saliva. Measurement of salivary PCT was thus undertaken using a commercial ELISA validated for use in serum, plasma and cell culture supernatants (Abcam, Cambridge) as well as the

automated assay used in the hospital laboratory (B.R.A.H.M.S Kryptor, Thermofisher Scientific, Hennigsdorf). A spike and recovery experiment was performed on saliva samples using the commercial ELISA.

5.6.2.1. Reagent and sample preparation

Saliva samples were obtained and stored as detailed in Methods (Section 2.5.1.3). A solution of PCT standard (55ng/ml) was prepared according to the manufacturer's instructions, and a serial dilution performed. Standard concentrations ranged from 27pg/ml to 20ng/ml. The supplied wash buffer, calibrator diluents, and substrate solutions were prepared according to the instructions provided.

5.6.2.2. ELISA procedure

The ELISA assay was performed on a supplied pre-coated 96-well PCT microplate. 100µl of prepared standard or sample was added to each well. Samples were assayed in duplicate. Standards and samples were incubated for 2.5 hours at room temperature before washing with the supplied wash buffer using an autowasher. 100µl of biotinylated PCT antibody was then added to each well and incubated for a further 1 hour. Following a further wash cycle, 100µl of HRP-Streptavidin conjugate was then added to each well, and incubated for a further 45 minutes. After washing, 100µl of TMB One-Stop Substrate Reagent was added and incubated for 30 minutes in a dark room, tapping the microplate to ensure mixing. 50µl Stop solution was then added to each well, and the optical density read by the ELx800 absorbance reader (Biotek instruments, Winooski) set to 450nm. As before, a 4 parameter logistic curve was constructed with each assay run, using the KC junior software package produced by Biotek.

5.6.2.3. Spike and recovery experiment

Following sample preparation, three randomly selected clinical saliva samples were serially diluted (neat, 1:2, 1:4) using the assay diluent provided. Each dilution was split into two 245 microlitre aliquots. One of each aliquot was spiked with a concentration of 400pg/ml PCT standard. Spiked and unspiked samples at each dilution were assayed in duplicate (Table 29).

Sample	Recovered spiked concentration (pg/ml)		Mean	Unspiked concentration (pg/ml)		Mean	Recovery (%)
A	223.91	253.72	238.82	9.88	54.76	32.32	53.21
A 1:2	461.59	497.54	479.57	158.04	150.41	154.22	83.83
A 1:4	443.41	434.96	439.19	136.71	73.09	104.90	86.13
B	107.72	114.14	110.93	<9	<9	0.00	28.58
B 1:2	395.05	379.11	387.08	285.66	192.79	239.23	38.10
B 1:4	554.65	478.25	516.45	196.33	237.30	216.81	77.20
C	282.51	321.16	301.84	183.86	218.82	201.34	25.89
C 1:2	485.16	578.83	532.00	340.76	358.59	349.67	46.98
C 1:4	490.67	397.93	444.30	252.10	269.83	260.96	47.24
Buffer	449.02	387.82	418.42	30.31	<9	15.16	97.03

Table 29: PCT concentrations measured in spiked and unspiked saliva samples at three dilutions.

Recovery of spiked PCT was poor in neat samples, and improved with dilution. Mean recovery of the 3 samples at 1:4 dilution was 70.2%. On the basis of this experiment, clinical saliva samples were diluted 1:4 for use in the assay.

5.6.3. Statistical methods

Statistical analysis was undertaken using R, version 3.1.3.

5.6.3.1. Univariate analysis

Univariate analysis of all clinical and biomarker variables was undertaken using logistic regression, where the outcome was SBI, and using multinomial regression, where the outcome was one of 'pneumonia', 'other SBI' or 'no SBI'.

5.6.3.2. Checking the assumptions of logistic regression

Explanatory variables were plotted against each other to look for evidence of co-linearity. Scatter plots and generalised additive model (GAM) plots[286] of continuous explanatory variables, fitted using the gam() function in the mgcv R package, were examined for evidence of non-linearity on the log-odds scale. Where the assumption of linearity appeared not to hold, we examined candidate variables using piecewise and polynomial transformations. Inspection of the GAM plot suggested a piecewise transformation of CRP should be used (Figure 16).

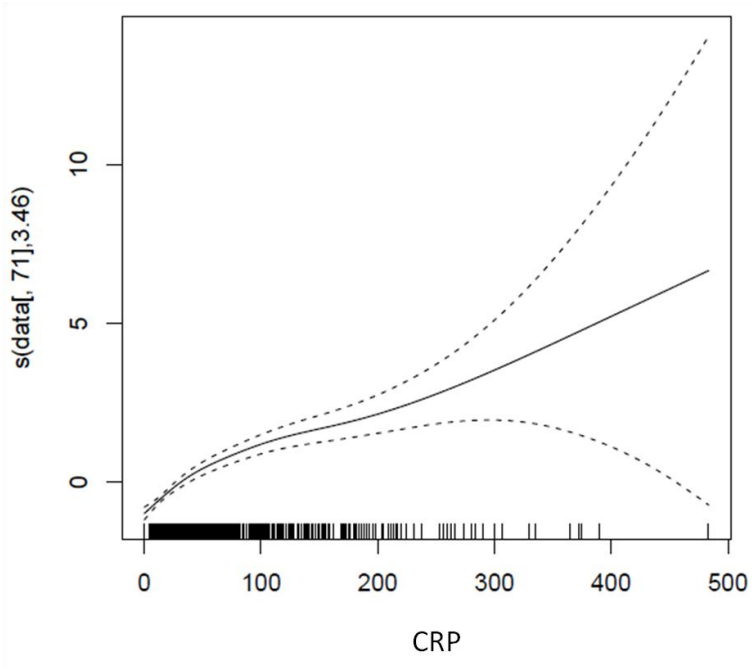


Figure 16: GAM plot of CRP versus SBI suggesting a non-linear relationship between CRP and SBI

5.6.3.3. Imputation of missing data

In order to provide valid inferences from incompletely recorded data, imputation of missing data was undertaken. Data was assumed to be "missing at random". Missing values were imputed ten times by fully conditional specification. This was performed with the 'multivariate imputation using chained equations' (MICE) package in R[269]. In this method, missing values are replaced by plausible values drawn from a conditional distribution specific to each individual predictor variable and defined by its own imputation model. Figure 17 illustrates the relationship between observed and imputed values of temperature in relation to heart rate for 10 imputations.

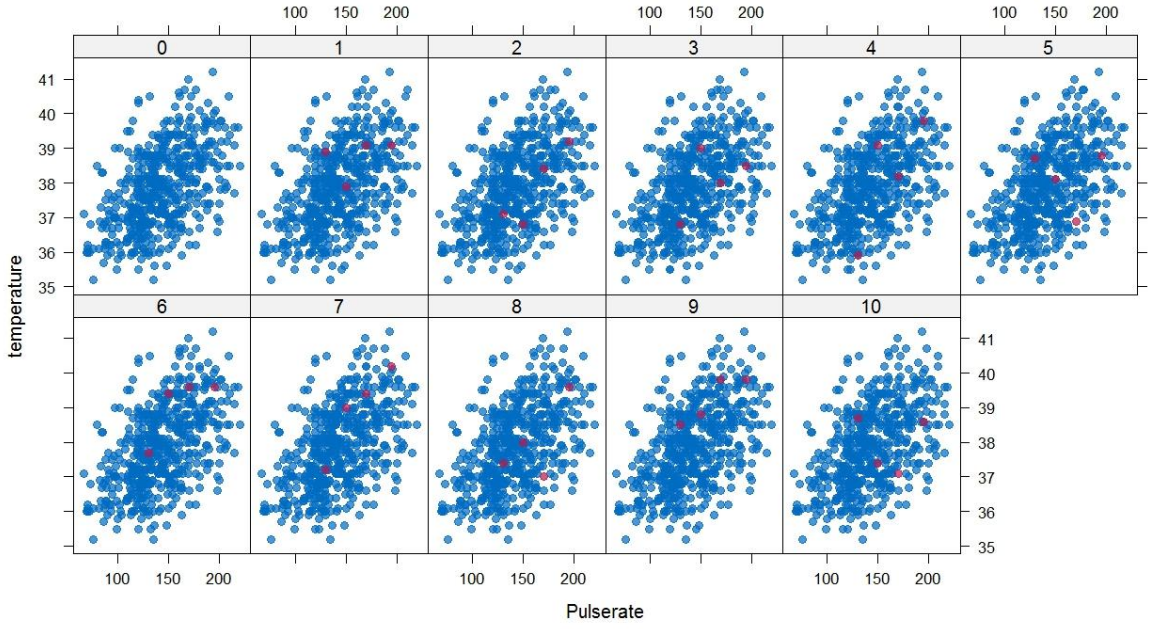


Figure 17: Observed (blue) and imputed (purple) values of temperature in relation to heart rate ('Pulserate') for 10 imputation cycles

5.6.3.4. Multivariable model for the diagnosis of SBI

The dataset was randomised into a split sample "derivation set", and "validation set". Following the initial univariate analysis, an estimate of the number of true null hypotheses was made using a plot of (1-p)-values[354] (Figure 18). Using this technique, the slope of the plot estimates the number of true null hypotheses to be rejected. We thus determined the significant explanatory variables to include in a forward stepwise model building process.

In the model building process, each of the significant explanatory variables was regressed against the outcome SBI. Improvement of the models from the null was tested by measuring the change in deviance between the null model and each univariate model. The variable associated with the most highly significant improvement in model deviance was then included in the model. The process was repeated, adding each remaining variable to this univariate model, and testing the significance of the change in deviance between each two variable model and that of the univariate model. This stepwise cycle of adding each remaining variable individually to the model established by the previous cycle was continued until the

addition of further explanatory variables produced no significant improvement in the residual deviance.

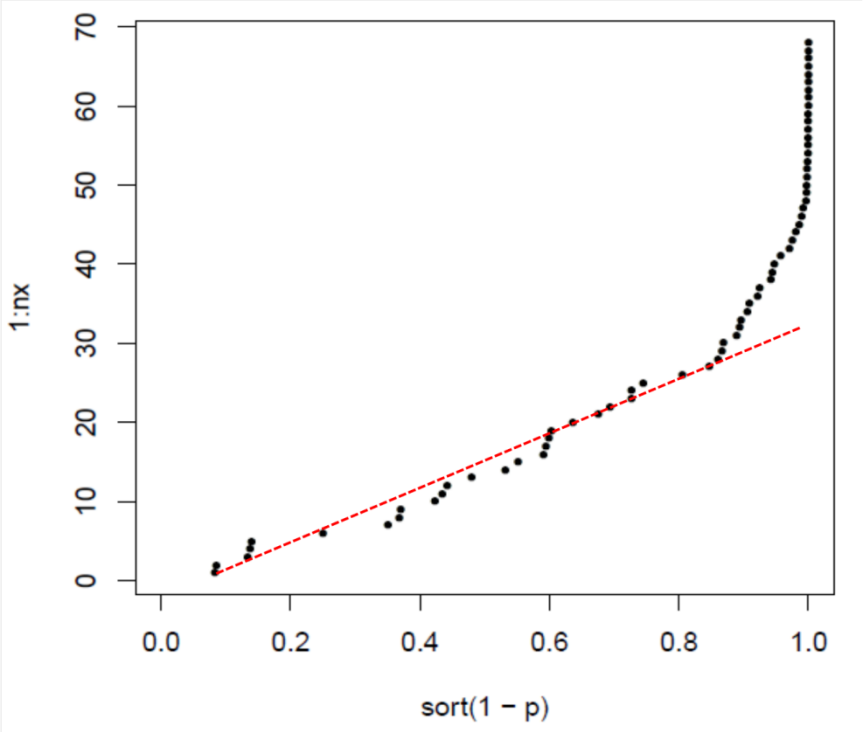


Figure 18: Plot of the sorted (1-p) values obtained from univariate logistic regression analysis of all variables for the outcome of SBI. The slope of the straight line section estimates the number of true null hypotheses[354].

Having identified a parsimonious model for the identification of SBI, these variables were then included in a multinomial logistic regression model using the categorical outcomes "pneumonia", "other SBI", and "no SBI". Multinomial regression was undertaken using the multinom () function in the 'nnet' package in R.

5.6.3.5. Model evaluation

Discrimination of the regression models was measured using the c statistic, and illustrated using Receiver Operating Characteristic (ROC) curves (roc() function of the 'pROC' package in R[320]). For the multinomial regression model, the c statistic estimated discrimination between pairs of patients – either a patient with pneumonia and a patient with no SBI, or patient with "other SBI" and a patient with

no SBI. Confidence intervals (95%) for the area under the ROC curve were estimated by means of a bootstrapping process using 2000 bootstrap replicates. Goodness-of-fit was tested using the Hosmer-Lemeshow test[355], and illustrated by calibration plots. The Hosmer-Lemeshow test sorts the study sample into deciles based upon predicted probabilities, and tests the significance of the difference between observed and expected frequencies using the chi-square distribution[274]. Influence plots were used to evaluate any excess contribution of individual study subjects to the fitted model. Performance characteristics of the models were calculated at various risk thresholds. Sensitivity, specificity, positive and negative likelihood ratios were estimated using the epiR package in R.

5.7. Results

Between 1st November 2010 and 3rd April 2012, 7949 children presented to the Alder Hey children's ED with fever, or history of fever as part of their presenting complaint. Of these 1872 had routine blood investigations carried out and were thus eligible for inclusion in the study. 1101 children were recruited to the study (see flowchart, Figure 19).

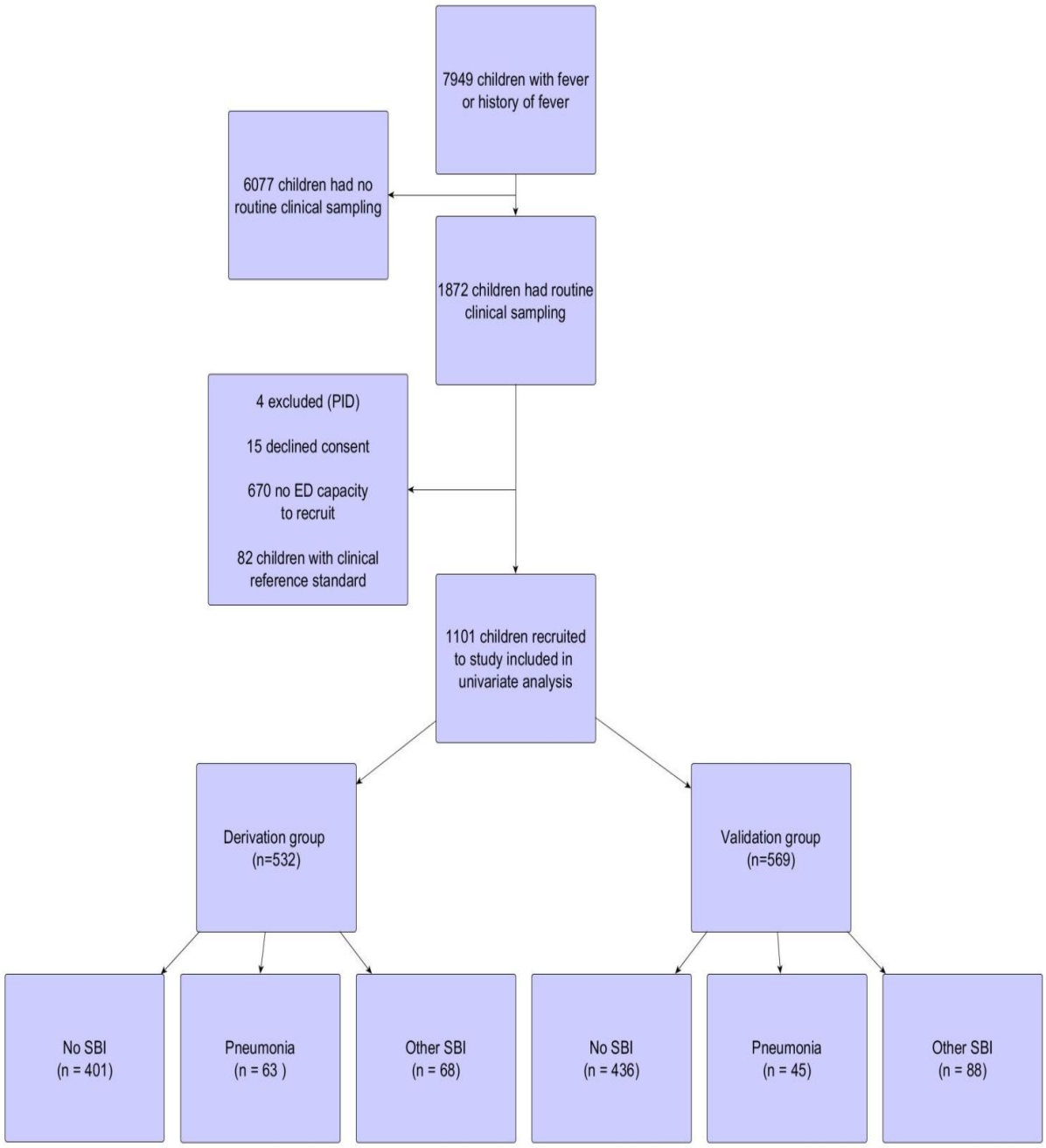


Figure 19: Study flow chart of children recruited to the SPICED study

5.7.1. Patient characteristics

The median age of children in the study was 2.4 years (IQR 0.9-5.7 years), 55% were boys. Approximately one third of children had significant comorbidities, requiring the ongoing care of a paediatric specialist. The characteristics of recruited children are described in Table 30.

A total of 264 children (24.0%) were diagnosed with SBI, 108 with pneumonia and 156 with other SBIs. Outcome diagnoses are illustrated in Figure 20. Nineteen children were admitted to PICU. Of these, nine had bacteraemia (seven MCD, one iGAS, and one *S.pneumoniae*), two of whom had concurrent meningitis (one MCD, and one *S.pneumoniae*). Five children were admitted to PICU with pneumonia including one child with empyema and Varicella Zoster Virus (chickenpox) infection. A single child with significant comorbidities was admitted to PICU with a *Pseudomonas* UTI. Four children were admitted with viral diagnoses, and in 3 of these cases PICU admission was for management of prolonged seizures. One young infant required ventilation for bronchiolitis (virus not identified) for a period of 6 days. A 10 month old previously healthy girl with *S.pneumoniae* meningitis and septicaemia was the only fatality observed during the course of the study.

Children with pneumonia were older than those with no SBI (3.5 years v 2.2 years, $p<0.001$), and more likely to have significant comorbidities (47% v 30%, $p<0.001$). Children with pneumonia and other SBIs had a higher median temperature and heart rate, than those with no SBI ($p<0.05$), and children with pneumonia had higher respiratory rates than those with no SBI (median 38 v 28, $p<0.01$).

	Overall n=1101		Pneumonia n=108		Other SBI n=156		No SBI n=837	
Demographics	Median	IQR	Median	IQR	Median	IQR	Median	IQR
Age	2.39	0.88-5.73	3.51†	1.60-6.29	2.28	0.43-7.54	2.21	0.92-5.35
	Proportion	95%CI	Proportion	95%CI	Median	IQR	Proportion	95%CI
Male sex	0.55	0.52-0.58	0.48	0.39-0.57	0.59	0.51-0.66	0.56	0.52-0.59
PMH	0.31	0.28-0.34	0.47†	0.38-0.57	0.26	0.19-0.33	0.30	0.27-0.33
Clinical variables	Median	IQR	Median	IQR	Median	IQR	Median	IQR
Temperature	37.8	37.0-38.6	37.9*	37.1-38.9	38.0*	37.2-38.8	37.7	36.9-38.6
Heart Rate	140	121-166	147*	132-170	148*	122-175	139	120-163
Respiratory Rate	30	24-38	38†	28-48	30	24-38	28	24-36
Biomarkers	Median	IQR	Median	IQR	Median	IQR	Median	IQR
CRP	19.6	5.8-54.0	49.0†	21.1-119	68.3†	28.9-137	14.3	4.0-36.5
WCC	11.5	7.9-15.8	11.8*	8.4-18.5	15.0†	10.9-20.5	10.8	7.7-14.7
NC	6.9	3.8-10.8	8.0†	4.8-13.4	10.0†	5.9-14.8	6.2	3.4-9.7
NGAL	77.1	52.5-121	92.1†	65.9-162	120†	74.4-170	69.7	49.5-103
PCT	0.23	0.10-0.83	0.49†	0.12-2.85	1.10†	0.15-5.85	0.18	0.09-0.53
Resistin	40.3	21.1-73.4	67.3†	31.4-107	60.6†	29.7-113	35.7	19.8-64.3
Outcomes	Median	IQR	Median	IQR	Median	IQR	Median	IQR
Length of stay	2	0-3	3†	2-6	4.5†	2-7	1	0-2
	n (%)	95%CI	n (%)	95%CI	n (%)	95%CI	n (%)	95%CI
PICU	19 (1.73)	1.11-2.68	5* (4.63)	2.00-10.4	10*(6.41)	3.52-11.4	4 (0.48)	0.19-1.22
Mortality	1 (0.09)	0.01-0.51	0	0-3.40	1 (0.65)	0.12-3.55	0	0-0.46

Table 30: Characteristics of study subjects. Statistical comparisons are between Pneumonia or Other SBI and No SBI. Continuous data were compared by means of the Kruskal Wallis test, proportions were compared by means of the Pearson's Chi squared statistic. Rare events such as admission to PICU or death were compared by means of a Monte Carlo simulation. †p<0.001 *p<0.05.

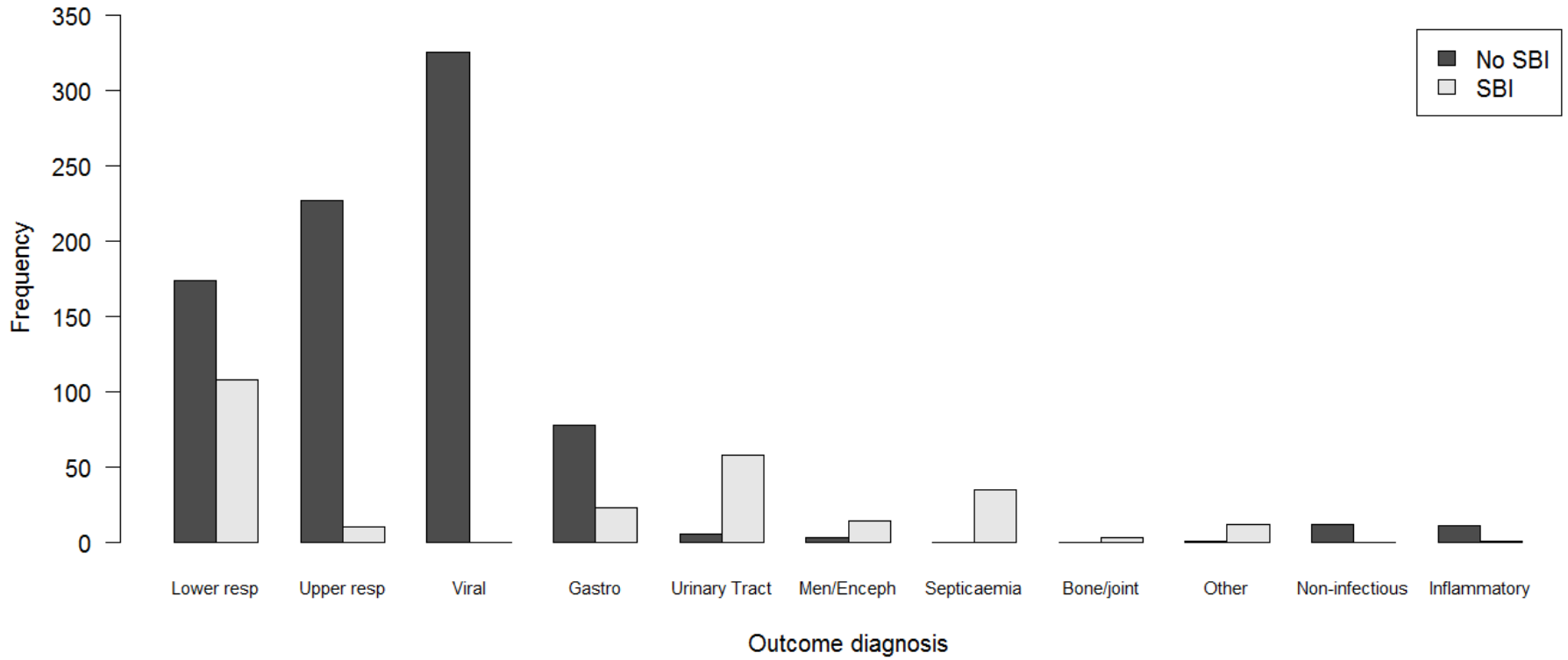


Figure 20: Outcome diagnoses in children recruited to the SPICED study by organ system

5.7.2. Salivary PCT in children

Saliva samples were obtained in 1006 children. The first 50 samples were batched and analysed using the B.R.A.H.M.S Kryptor system, validated for use in plasma. Of these, 6 samples were insufficient for analysis, and of the remaining 44 all but two failed to detect PCT (lower limit of assay detection 20 pg/ml). The two samples with detectable PCT were quantified at 60 pg/ml, and 210 pg/ml. The range of PCT in the corresponding plasma samples (collected contemporaneously) was up to 17.6 ng/ml. All but three had plasma levels of PCT >60 pg/ml.

Following the demonstration of measurable quantities of PCT in spiked samples using a commercial ELISA (Abcam, Cambridge), a further 48 clinical samples were tested in duplicate. Of these, a single sample yielded a result of 540 pg/ml, while the remainder were undetectable (<160 pg/ml). We concluded that using two independent methods of detection, we were unable to detect significant levels of PCT in saliva.

5.7.3. Univariate analysis

Initial univariate analysis was undertaken in the entire dataset (n=1101). Consistent with other studies, increased work of breathing (odds ratio (OR) 10.41, 95% confidence interval 6.69 to 16.2), hypoxia (9.29, 5.35 to 16.1), and other features of an abnormal respiratory examination were significantly associated with the presence of pneumonia. These features reduced the likelihood of other SBIs. Neck stiffness (20.6, 2.1 to 199), a bulging fontanelle (3.37, 1.35 to 8.40), irritability (3.23, 1.54 to 6.78) and dysuria (3.34 , 1.28 to 8.68) were all associated with other SBIs. Prolonged capillary refill time was associated with other SBIs (1.43, 1.05 to 1.97) but not pneumonia. Neither an ill appearance nor parental concern predicted pneumonia nor other SBIs. (See Table 32 and Figure 21).

The likelihood of pneumonia and other SBIs increased linearly with heart rate, respiratory rate and temperature. The biomarkers CRP, Procalcitonin, NGAL and Resistin were all associated with likelihood of SBI. Lactate and blood glucose were not, though were measured in fewer children (Table 31).

Biomarkers	n	Pneumonia			Other SBI		
		OR	LCI	UCI	OR	LCI	UCI
Procalcitonin	1034	1.22	1.15	1.29	1.23	1.16	1.30
Neutrophils	1059	1.09	1.06	1.13	1.12	1.09	1.15
WCC	1059	1.05	1.02	1.08	1.08	1.06	1.11
CRP	1072	1.02	1.01	1.02	1.02	1.02	1.02
Resistin	1045	1.01	1.00	1.01	1.01	1.01	1.01
NGAL	1046	1.00	1.00	1.01	1.01	1.00	1.01
Blood glucose	123	0.78	0.54	1.12	1.03	0.89	1.20
Lactate	167	0.67	0.42	1.09	1.12	0.84	1.50

Table 31: Odds ratios of biomarker variables in univariate multinomial regression for the diagnoses of pneumonia and other SBIs. ‘n‘: number of subjects in which the variable was recorded. OR: Odds ratio, LCI: lower 95% confidence interval, UCI: upper 95% confidence interval

Variable	n	Pneumonia			Other SBI		
		OR	LCI	UCI	OR	LCI	UCI
Neck stiffness	652	0	0	$3x10^{23}$	20.6	2.13	199
Normal air entry	1069	0.09	0.06	0.14	9.60	2.34	39.4
Chest clear	1069	0.09	0.06	0.14	5.79	2.33	14.4
Bulging fontanelle	246	0.16	0.04	0.62	3.37	1.35	8.40
Rash	1009	0.30	0.17	0.50	0.50	0.33	0.77
Abdominal pain	306	0.42	0.19	0.93	1.83	0.93	3.59
Parental concern	159	0.72	0.18	2.84	0.47	0.15	1.49
History of myalgia	151	0.73	0.22	2.41	0.73	0.18	2.85
Irritability	256	0.84	0.32	2.22	3.23	1.54	6.78
Abnormal ENT signs	921	0.84	0.54	1.31	0.45	0.30	0.68
Heart rate	1058	1.01	1.00	1.02	1.01	1.00	1.01
History of diarrhoea	899	1.03	0.62	1.72	0.55	0.33	0.92
Respiratory rate	907	1.05	1.04	1.07	1.00	0.99	1.02
Duration of fever (day)	1101	1.09	1.05	1.13	0.89	0.83	0.96
Temperature	1092	1.23	1.03	1.47	1.22	1.05	1.42
Prolonged Capillary Refill (>2s)	909	1.37	0.63	3.02	2.08	1.15	3.75
History of dysuria	270	1.53	0.32	7.32	3.34	1.28	8.68
Dehydration	480	1.84	0.91	3.73	2.06	1.12	3.78
Pallor	469	1.90	1.03	3.51	2.67	1.53	4.66
Comorbidity	1101	2.12	1.42	3.19	0.82	0.56	1.21
History of drowsiness	295	2.30	0.90	5.92	2.25	0.93	5.42
Prior antibiotics	1097	2.55	1.66	3.93	1.19	0.78	1.82
Wheeze	1074	3.21	1.84	5.60	0.20	0.05	0.83
Ill appearance	108	3.56	0.71	17.7	2.22	0.41	12.09
History of chest pain	118	7.75	2.06	29.1	0.01	$2x10^{-32}$	$1x10^{26}$
Chest crackles	1071	8.16	5.30	12.57	0.20	0.07	0.56
History of cough	847	9.00	3.88	20.9	0.22	0.14	0.35
Hypoxia (Sats <92%)	963	9.29	5.35	16.10	0	0	$1x10^{18}$
Decreased Breath Sounds	1068	10.00	6.25	16.00	0.10	0.01	0.73
Increased Work of Breathing	1071	10.41	6.69	16.20	0.64	0.36	1.13

Table 32: Odds ratios of clinical variables in univariate multinomial regression analysis for the outcomes of pneumonia and other SBIs. 'n': number of subjects in which the variable was recorded. ENT: ear, nose, throat. OR: Odds ratio, LCI: lower 95% confidence interval, UCI: upper 95% confidence interval Variables associated with an increased risk are highlighted red, and those associated with a reduced risk are highlighted green.

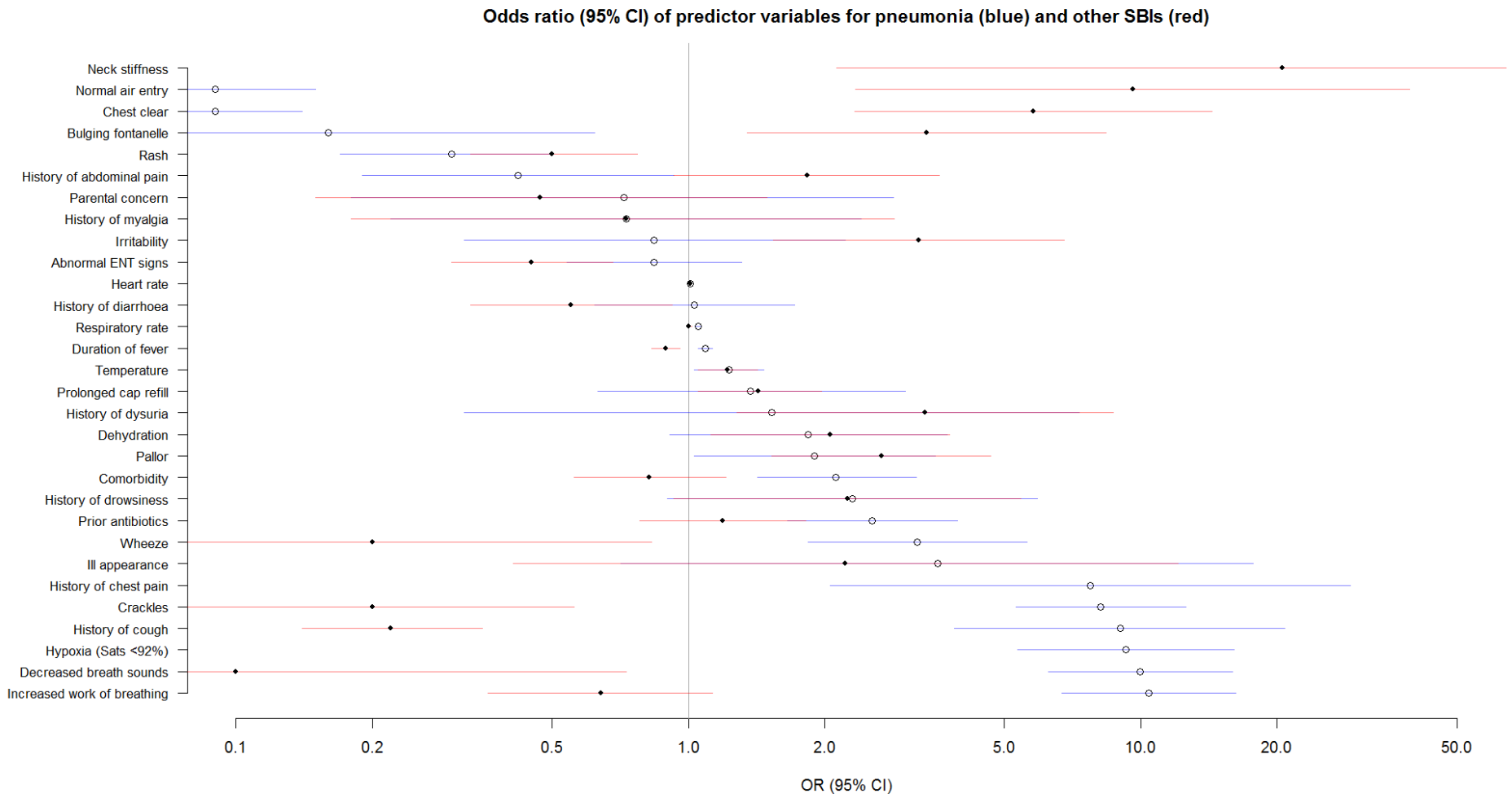


Figure 21: Plot of odds ratios (with 95% confidence intervals given by horizontal bars) of clinical variables in univariate multinomial regression analysis for the outcomes of pneumonia and other SBIs.

The presence of a rash reduced the likelihood of both pneumonia and other SBIs. The presence of a petechial rash was associated with a reduced risk of both pneumonia and other SBI (Table 33).

Rash (n=937)	n	Pneumonia			Other SBI		
		OR	LCI	UCI	OR	LCI	UCI
None	570		Ref			Ref	
Maculopapular	112	0.57	0.28	1.15	0.58	0.30	1.15
Petechial	209	0.17	0.07	0.39	0.33	0.18	0.61
Purpuric	10	0	0	Inf	1.21	0.25	5.82
Vesicular	18	0.87	0.19	3.97	1.60	0.51	5.14
Other	18	0	0	Inf	0.65	0.65	2.89

Table 33: OR for the presence of rash in univariate multinomial regression

5.7.4. Multivariable risk prediction model for the diagnosis of SBI

Using a forward stepwise model building approach, a multivariable logistic regression model was fitted to the derivation group (n=532) for the outcome of SBI. The model derived was:

$$\text{logit (SBI)} = -1.676027 + 0.034923\ (\text{CRP.low}) + 0.011681\ (\text{CRP.high}) + 0.030766\ (\text{resp.rate}) + 0.164464\ (\text{PCT}) + (-1.311112)*(\text{Normal.air.entry}) + 0.003741\ (\text{Resistin})$$

The coefficients of the derived multivariable model for the diagnosis of SBI are reported in Table 34.

Variable	beta	SE	z value	p-value	OR	LCI	UCI
(Intercept)	-1.676	0.490	-3.417	0.001	0.187	0.070	0.485
CRP (<30mg/l)	0.035	0.013	2.662	0.008	1.036	1.010	1.063
CRP (>30mg/l)	0.012	0.003	3.425	0.001	1.012	1.005	1.019
Respiratory rate	0.031	0.011	2.828	0.005	1.031	1.010	1.054
PCT	0.164	0.049	3.383	0.001	1.179	1.082	1.311
Normal air entry	-1.311	0.284	-4.617	0.000	0.270	0.154	0.470
Resistin	0.004	0.002	2.128	0.033	1.004	1.000	1.007

Table 34: Coefficients of the multivariable model for the diagnosis of SBI in the derivation group.

In the derivation group, discrimination measured by the c statistic was 0.77 (95% CI 0.72-0.82). Calibration appeared acceptable (HL test, p=0.79), though the model predicted a higher rate of SBI than was observed in low-risk children (Figure 22).

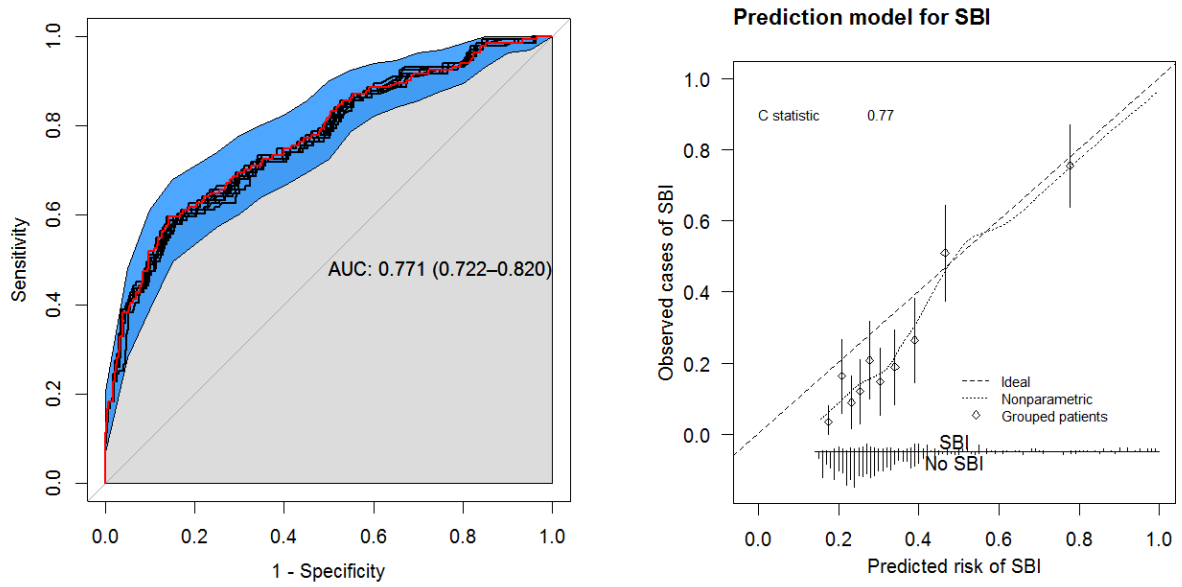


Figure 22: ROC curve (left) and calibration plot (right) for the multivariable model for SBI in the derivation group. ROC curves are plotted using the linear predictor generated by each of the 10 imputed datasets, and the mean is shown in red. The blue shaded area represents the 95% CI of the ROC curve obtained by 2000 bootstrap replicates

5.7.5. Internal validation of the SBI prediction model

Discrimination of the model remained acceptable when applied to the validation group (C statistic 0.79 (95% CI 0.75-0.84). Calibration of the model was good (Figure 23).

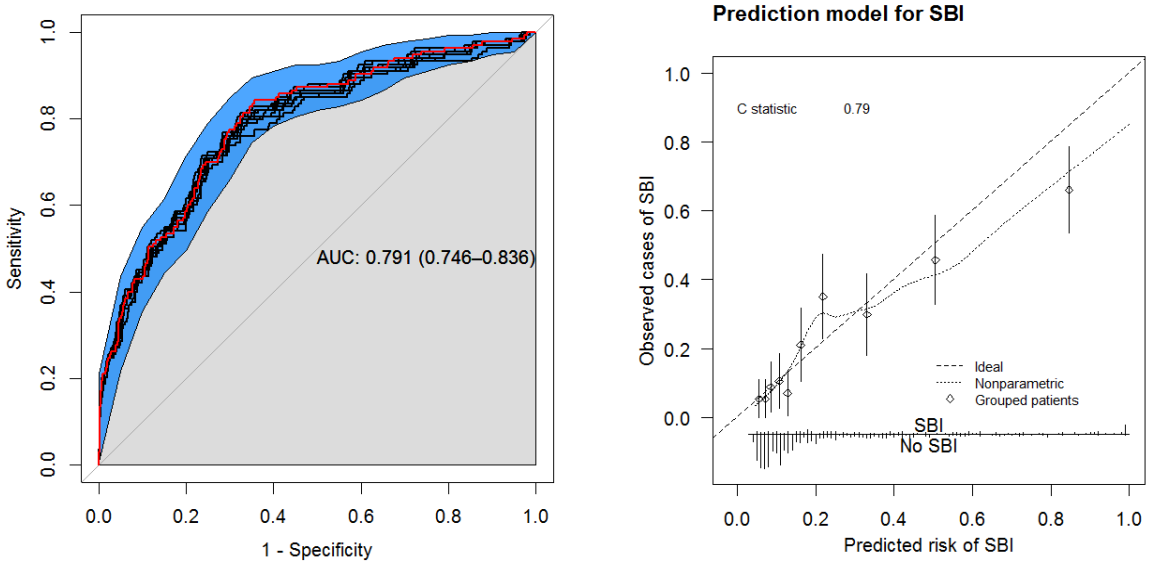


Figure 23: ROC curve (left) and calibration plot (right) for the multivariable model for SBI in the validation group. ROC curves are plotted using the linear predictor generated by each of the 10 imputed datasets, and the mean is shown in red. The blue shaded area represents the 95% CI of the ROC curve obtained by 2000 bootstrap replicates.

5.7.6. Derivation of multinomial risk prediction model

Having established a parsimonious model for the diagnosis of SBI, these variables were fitted to a multinomial logistic regression model for the categorical diagnosis of pneumonia, other SBI or no SBI.

The coefficients of the multivariable model for the diagnosis of pneumonia and other SBIs are documented in Table 35. The model discriminated well between pneumonia and no SBI, with a C statistic of 0.89 (95% CI 0.84-0.93) and between other SBIs and no SBI (C statistic 0.79, 95% CI 0.73-0.86). Calibration of the model for pneumonia was good, though the model appeared to predict fewer cases of other SBI than were observed (Figure 24).

	Pneumonia				Other SBI			
	Est	OR	LCI	UCI	Est	OR	LCI	UCI
(Intercept)	-2.516	0.081	0.025	0.260	-2.779	0.062	0.016	0.239
CRP (<30)	0.025	1.025	0.990	1.060	0.045	1.046	1.011	1.081
CRP (>30)	0.010	1.010	1.003	1.018	0.012	1.012	1.005	1.019
Respiratory rate	0.047	1.048	1.021	1.076	0.009	1.009	0.980	1.039
PCT	0.173	1.189	1.079	1.310	0.168	1.183	1.074	1.303
Normal air entry	-2.387	0.092	0.046	0.182	0.240	1.271	0.514	3.142
Resistin	0.003	1.003	0.999	1.008	0.004	1.004	1.000	1.007

Table 35: Coefficients of the multivariable model using multinomial regression for the diagnosis of pneumonia, other SBIs and no SBI.

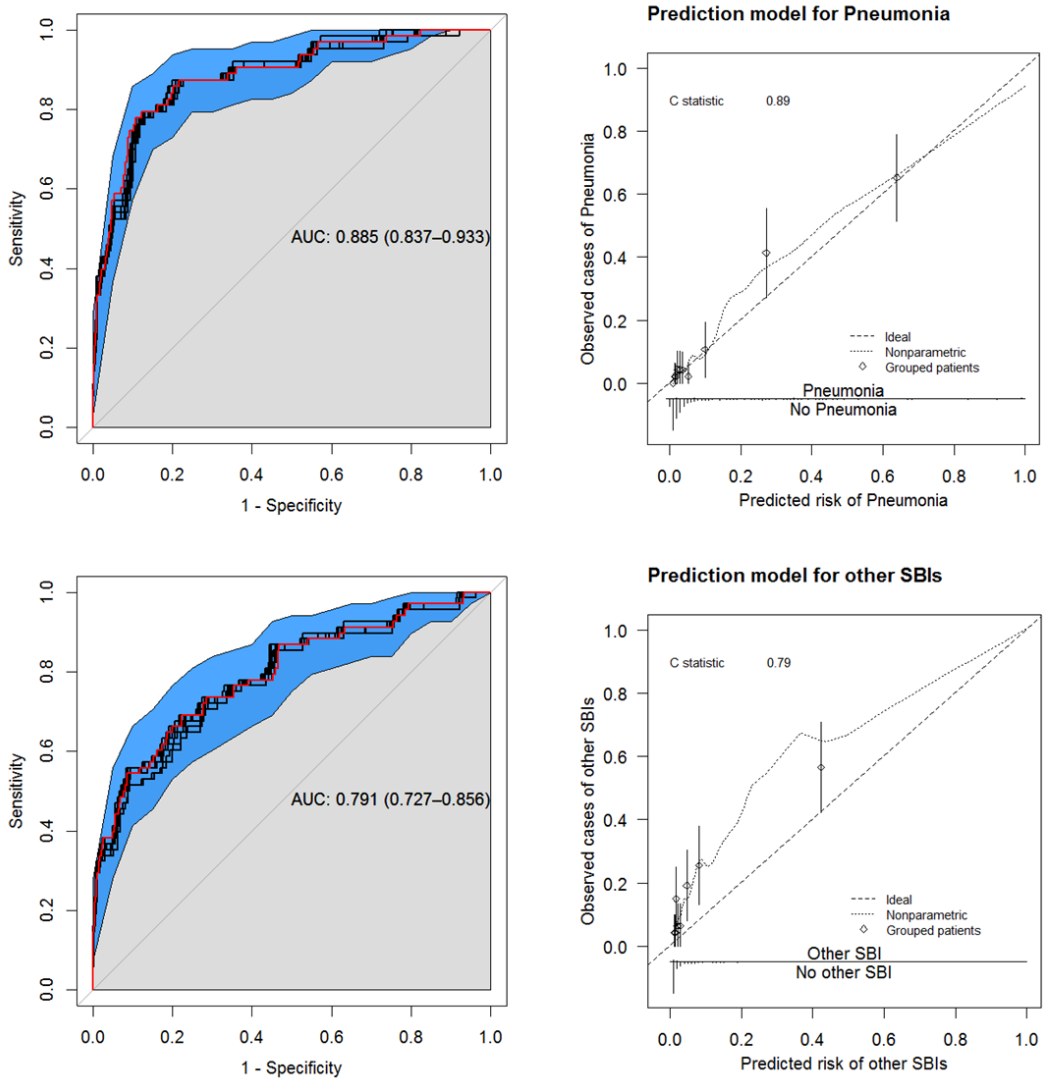


Figure 24: ROC curves (left) and calibration plots (right) for the multivariable risk prediction models for pneumonia (top) and other SBIs (bottom) in the derivation group.

5.7.7. Internal validation of multinomial risk prediction model

The derived multinomial model was then applied to an internal validation group (n=569). For the diagnosis of both pneumonia, and other SBI, discrimination of the model was good (AUC 0.84, 95%CI 0.78 to 0.90 and 0.77, 95%CI 0.71 to 0.83 respectively. Calibration plots revealed that the model overestimated the risk of pneumonia and underestimated the risk of other SBI in the validation group (Figure 25).

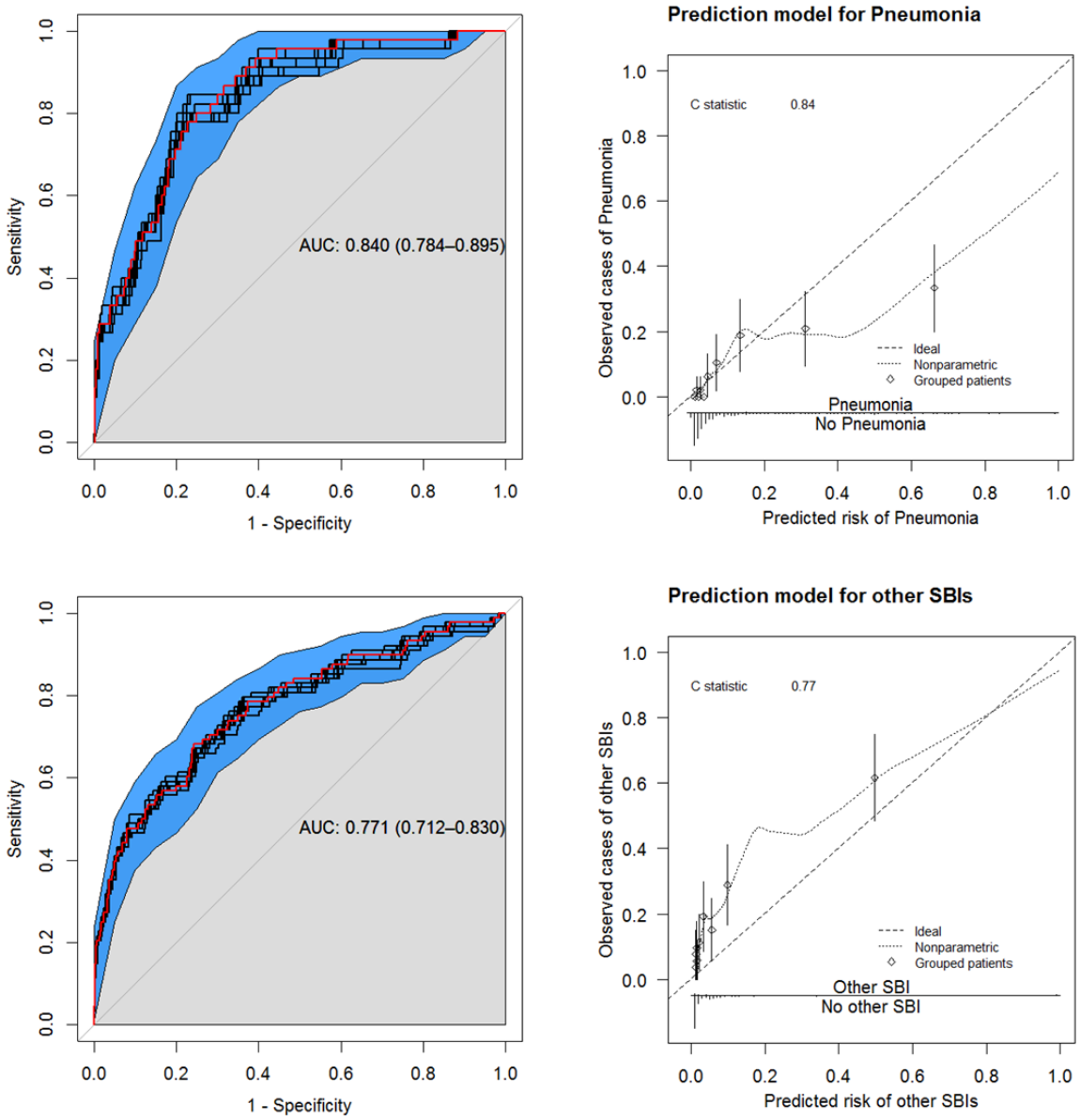


Figure 25: ROC curves (left) and calibration plots (right) for the multivariable risk prediction models for pneumonia (top) and other SBIs (bottom), applied to the validation group.

Performance characteristics for the multinomial model were calculated at various risk thresholds (Table 36). At a low-risk threshold of 5%, the model performed well in ruling out pneumonia (NLR 0.10, 95% CI 0.03 to 0.31), though was less good at ruling out other SBIs (NLR 0.28, 0.15 to 0.51). At a high-risk threshold, the models appeared better able to rule in other SBIs than pneumonia. A risk threshold of 20% achieved a PLR of 5.14 (3.81 to 6.94) for the diagnosis of other SBIs, and of 3.82 (2.80 to 5.22) for the diagnosis of pneumonia.

Pneumonia												
	Sensitivity	95% CI	Specificity	95% CI	PPV	95% CI	NPV	95% CI	PLR	95% CI	NLR	95% CI
2.5%	1	(0.88 - 1)	0.37	(0.32 - 0.41)	0.14	(0.10 - 0.18)	1	(0.97 -1)	1.58	(1.47 - 1.70)	0	NA
5%	0.93	(0.82 - 0.99)	0.64	(0.59 - 0.69)	0.21	(0.16 - 0.27)	0.99	(0.97 - 1)	2.59	(2.24 - 3.00)	0.10	(0.03 - 0.31)
10%	0.80	(0.65 - 0.90)	0.76	(0.72 - 0.80)	0.26	(0.19 - 0.34)	0.97	(0.95 - 0.99)	3.38	(2.71 - 4.23)	0.26	(0.15 - 0.47)
20%	0.62	(0.47 - 0.76)	0.84	(0.80 - 0.87)	0.28	(0.20 - 0.38)	0.96	(0.93 - 0.97)	3.82	(2.80 - 5.22)	0.45	(0.31 - 0.66)
30%	0.47	(0.32 - 0.62)	0.89	(0.86 - 0.92)	0.30	(0.20 - 0.43)	0.94	(0.91 - 0.96)	4.23	(2.81 - 6.39)	0.60	(0.46 - 0.79)
Other SBI												
	Sensitivity	95% CI	Specificity	95% CI	PPV	95% CI	NPV	95% CI	PLR	95% CI	NLR	95% CI
2.5%	1	(0.94 - 1)	0.00	(0 - 0.02)	0.16	(0.14 - 0.20)	1	(0.28 - 1)	1.00	(1.00 - 1.02)	0	NA
5%	0.89	(0.80 - 0.94)	0.41	(0.36 - 0.45)	0.23	(0.19 - 0.28)	0.95	(0.90 - 0.97)	1.49	(1.34 - 1.66)	0.28	(0.15 - 0.51)
10%	0.77	(0.67 - 0.86)	0.66	(0.61 - 0.70)	0.31	(0.25 - 0.38)	0.94	(0.90 - 0.96)	2.28	(1.91 - 2.71)	0.34	(0.23 - 0.51)
20%	0.63	(0.52 - 0.73)	0.88	(0.84 - 0.91)	0.51	(0.41 - 0.61)	0.92	(0.89 - 0.94)	5.14	(3.81 - 6.94)	0.43	(0.33 - 0.56)
30%	0.47	(0.36 - 0.58)	0.94	(0.91 - 0.96)	0.60	(0.48 - 0.72)	0.90	(0.87 - 0.92)	7.52	(4.90 - 11.6)	0.57	(0.47 - 0.69)

Table 36: Performance characteristics of the multinomial model for the prediction of pneumonia (top) and other SBIs (bottom) in the validation set.

5.8. Discussion

5.8.1. Main findings

This chapter details the findings of a large, prospective study of children up to 16 years of age presenting to the Emergency Department with febrile illness. In this heterogeneous group, risk prediction for SBIs is possible.

5.8.1.1. Univariate analysis

Initial univariate analysis of our large sample reveals associations broadly in line with other published data[6,10,96]. Our finding that clinical variables such as hypoxia, abnormal respiratory findings, irritability and dehydration increase the likelihood of SBI is consistent with studies from similar settings[10,96,97]. We failed to demonstrate the value of more subjective assessments, such as an 'ill appearance', and 'parental concern'. Recent studies which have quantified the value of an 'ill appearance' in the febrile child in ED have concluded that it is of limited value[96,97], though in the largest recent study of clinical features of SBI in febrile children, it was of greater value in predicting the most serious outcome of bacteraemia[6]. In the low-risk setting of primary care, parental concern that the current illness is different to previous illnesses is strongly associated with the presence of serious infection, as is the clinician assessment that 'something is wrong'[356]. This 'gut feeling' of the clinician that something is wrong, particularly when at odds with a reassuring clinical assessment may have diagnostic value in this setting[357]. Whilst we may have failed to consistently apply these variables, because our study made use of numerous clinicians with their own understanding of these assessments, we believe this to be a 'real-life' – and thus plausible – illustration of their true value. There was, however, also a significant problem of missing data for some of these more subjective assessments, and it is possible that we failed to accurately quantify their value.

A 'non-blanching' rash in the context of the febrile child has long been considered a 'red flag' sign, and remains so in the latest NICE guidelines for the management of fever in children under 5 years[184]. Of interest is the finding that, in univariate analysis, a petechial rash (non-blanching spots less than 2mm in diameter) is

associated with a reduced risk of both pneumonia and other SBIs (see Table 33). This requires some explanation. Firstly, the number of children included in the study with a petechial rash was high - moreso than, for example, a maculopapular rash. This is a consequence of a specific departmental protocol advocating blood testing in all children with fever and a petechial rash. As a result, many clinically well children were tested, and became eligible for the study. Upon adjustment for other indicators of clinical wellbeing in the multivariable model, this association disappears.

The particular concern of the clinician with regard to a non-blanching rash in a febrile child is the possibility of septicaemia, and particularly (though not exclusively) meningococcal septicaemia. The high number of respiratory and urinary tract infections in our sample may have masked the association of a petechial rash with less common outcome diagnoses including bacteraemia. Multivariable modelling for bacteraemia was not robust as a result of its relative infrequency (n=35). Univariate analysis however suggests that the presence of a petechial rash was associated with a small increase in the likelihood of bacteraemia (OR 2.58, 95% CI 1.1 to 5.99). This translates into a PLR of 1.52 (95%CI 0.92 to 2.50) increasing the probability of bacteraemia from a pre-test probability in our sample of 3.2% to a post-test probability of 5.3%. Of 12 children in the study with a final diagnosis of MCD, 10 had a rash, of which 5 had petechiae, 3 purpura and 2 a maculopapular rash.

A recent large prospective study of febrile children in ED with a low-risk of SBI failed to demonstrate an association between 'rash' and bacteraemia[6]. In a small recent study of febrile children presenting to the ED with petechial rash, a viral infection was identified in 39/58 children, most commonly the herpesviruses (EBV, CMV), enteroviruses, rhinovirus and other respiratory viruses (including RSV), and adenovirus. No cases of bacteraemia were identified, though 5 children had 'presumed sepsis'[358]. Even in the high-risk setting of unwell children with presumed meningococcal disease, a substantial proportion are found to have alternative diagnoses[359]. Non-blanching rashes, including petechial rashes may of course indicate serious illness, including SBI. As with other predictive clinical signs, the

presence of petechial spots should prompt a thorough clinical assessment, which may include a period of observation, and further investigation. Specifically in well appearing children in the era of conjugate vaccines, the value of this sign may be overstated however.

5.8.1.2. Multivariable risk modelling

A stepwise model building approach yielded a parsimonious model for the diagnosis of SBI which performed well in the validation group. The model made use of commonly collected clinical signs alongside a combination of biomarkers. Using these same variables we derived and validated a multinomial model which discriminated well between pneumonia, other SBIs and no SBI. In the validation group, the models predicted a higher rate of SBI - in particular, a higher rate of pneumonia - than was observed. This was reflected in the relative lack of specificity for the pneumonia model, even at high-risk thresholds (specificity 89% at a risk threshold of 30%, PLR 4.2).

5.8.2. Strengths

This is a large prospective study designed to derive and internally validate a prediction model comprising clinical and biomarker variables for the diagnosis of SBI in the children's ED. We have measured multiple biomarkers of SBI in more than 1000 children. We have evaluated children from birth to 16 years of age, irrespective of past medical history or clinical syndrome at presentation, and obtained comparable discrimination to other studies. In common with other recent data [6,96], we have demonstrated the value of a diagnostic prediction model combining clinical and biomarker variables.

5.8.3. Limitations

The study is a single centre study, and whilst we have performed internal validation of our derived model, external validity requires the demonstration of its value in an alternative setting. Our approach of using a split sample for internal validation may underestimate the true value of the diagnostic model, though is a robust and well established methodology[360].

Diagnostic studies of this kind with imperfect reference standards require a pragmatic approach in order to determine outcomes. Diagnostic tests were requested at the discretion of the clinical team, and not all tests were performed in all children. This is a well-recognised limitation of studies in this context. In particular, an aetiological diagnosis of lower respiratory tract infections is challenging. We recorded only 63 positive samples for respiratory pathogens. In view of the fact that approximately 500 children received an outcome diagnosis of either an upper or lower respiratory infection, a universal application of respiratory pathogen detection assays may have yielded additional evidence upon which to base our classification. Our use of a radiological diagnosis of 'pneumonia', despite its limitations, is commonly used in this setting[9,251], though some have required two independent radiological opinions. Despite these limitations, we believe that our approach, using a pre-defined composite reference standard is robust. By establishing clear criteria for each outcome diagnosis in advance we hope to minimise verification bias.

As with other similar studies, we have only studied children already considered at risk of SBI, in whom the clinical team have initiated further investigation. Inevitably then, the overall risk of SBI in the study sample (24%) is substantially higher than that of all febrile children attending the ED. It remains necessary to undertake diagnostics research in low-risk populations (that of all children attending the ED, or in primary care)[8]. With the increasing acceptance of near patient testing, evaluating clinical prediction and decision tools using biomarkers in these groups is possible, and may have an important role in improving rational decision-making. The difficulty of undertaking such evaluations is not insubstantial however. Appropriate outcomes of interest need to be identified as the lack of extensive investigation precludes the use of robust outcome diagnoses, whilst the reliable collection of clinical data in the low-risk, high turnover setting is challenging. A large recent study of almost 10000 febrile children in primary care concluded that current CPRs derived in the ED failed to reliably predict the need for referral to hospital. Many of the clinical variables included in the CPRs were not routinely documented in the primary care setting[361].

The process of model building was undertaken in a forward stepwise manner, testing the improvement in model fit at each stage (by measuring the change in residual deviance). With this approach a simple, parsimonious model was achieved which included three biomarkers and two clinical variables. This process of stepwise model building is well-established in the context of clinical risk prediction models, but has been criticised for failing to identify important predictor variables from candidate variables, particularly where there is significant collinearity. Furthermore, stepwise variable selection may lead to biased regression coefficients, and overestimate precision, leading to falsely narrow confidence intervals[362]. The derived model, while simple to implement, may in addition lack the necessary face validity to be considered of clinical value. Clinicians may question the omission of clinical variables recognised to have an important association with the presence of serious infection. A theoretically satisfying response to each of these concerns is achieved with the inclusion of all variables determined to be important based upon clinical expertise.

5.8.4. Next steps

These results support a growing body of research to suggest that clinical prediction models can improve the prediction of risk of SBI in febrile children of all ages presenting to the ED. The derived models presented here require external validation in alternative populations before undergoing impact analysis to determine their value in improving decision-making in the clinical setting[363].

Our model building approach driven by associations in the data yielded a simple model making use of only two clinical variables alongside a combination of biomarkers. This is a plausible model which performs well in the validation set. The inclusion of two clinical signs relating to the respiratory system likely reflects the preponderance of respiratory infections in this setting. This process of model selection is open to criticism, particularly in the context of multinomial regression[362]. Our internal validation provides some reassurance of the appropriateness of the model, but as already stated, external validation is now necessary. An alternative to this approach would be to include a larger set of clinically relevant variables based upon the published literature irrespective of their apparent statistical contribution to the model.

Two recent diagnostic accuracy studies published the results of the use of multivariable CPRs in febrile children presenting to the ED[9,96]. A further study proposed the use of a combination of biomarker tests (CRP, PCT and urinalysis in the 'Lab score') and has performed well on external validation[7,253,364]. This dataset provides the opportunity to validate these rules in an external population, and also to examine the additional value of the multiple biomarkers measured in our population. This shall be the focus of the next chapter.

5.9. Conclusions

A diagnostic model combining clinical and biomarker variables discriminated well between pneumonia, other SBIs and no SBI in febrile children presenting to the ED. Improving the ability of clinicians to discriminate between SBI and self-limiting infections has the potential to improve rational decision-making by initiating prompt therapy in those with SBI, and safely discharging those without. For this potential to be realised, risk prediction rules such as this need to be externally validated before impact analysis is undertaken of decision rules designed to incorporate this improved risk prediction.

Chapter 6: External validation of risk prediction models and comparison using net reclassification improvement

6.1. Overview of the chapter

In the previous chapter, a multivariable clinical prediction model discriminated well between pneumonia, other SBI and no SBI, and performed well on internal validation. In order to realise the clinical value of improved clinical risk prediction, such models are required to undergo a process of external validation. In this chapter, I will use the dataset obtained during the SPICED study to externally validate three previously published clinical prediction models (by Brent[9], Galetto-Lacour[7] and Nijman[96]). Using Net Reclassification Improvement, I will also estimate improvements in classification between models.

6.2. Background

6.2.1. The current approach to the febrile child in the ED

Numerous studies have estimated the diagnostic accuracy of individual clinical signs[10] and laboratory tests[185] in the diagnosis of SBI in children. Recognising the limitations of such signs in isolation, the NICE 'feverish illness in children' guideline[184], sought to combine clinical signs to improve the ability of clinicians to recognise serious infections. Though widely implemented in the UK, recent evaluations of the NICE guideline have highlighted its own limitations. In a study of more than 15000 febrile children under 5 years, the presence of any intermediate ("yellow") or high-risk ("red") signs resulted in a sensitivity of 86%, with a specificity of 28% for the diagnosis of SBI. The overall *c* statistic, measuring discrimination between SBI and no SBI was 0.64[250]. A subsequent analysis applying the NICE guideline to a further 6000 children recruited prospectively to seven studies in 'ambulatory care' settings (primary care or ED), found that few of the individual signs had value in ruling in serious infection in more than one dataset, and in no dataset was the guideline able to rule out serious infection[352].

In the UK, the most commonly used tests to support the diagnosis of SBI in children are the White Cell Count (WCC), and CRP. A large recent evaluation of the WCC highlighted its limited value in this context, with a *c* statistic of 0.65 for the diagnosis of SBI. At a threshold of $15x10^9$/l, the test achieved a positive LR of 1.93 (95% CI 1.75 to 2.13), and a negative LR of 0.70 (0.65 to 0.75)[186]. CRP has been

evaluated more favourably. A meta-analysis which included nearly 1400 children in 5 studies estimated a pooled positive LR of 3.15 (95% CI 2.67 to 3.71) and pooled negative LR of 0.33 (0.22 to 0.49)[185]. CRP is advocated by the NICE clinical guideline for use in children identified as being at risk of SBI by the 'traffic light' evaluation[184].

I have previously described the markers PCT, Resistin, and NGAL and their potential value as diagnostic biomarkers of SBI in the children's ED (Chapter 1.6.6). Compared to CRP, PCT has favourable performance in the diagnosis of SBI in children, particularly in the early stages of infection[101,189,190]. In combination PCT, Resistin and NGAL improved the diagnosis of pneumonia in Malawian children with pneumonia or meningitis[215]. In Chapter 5, both PCT and Resistin (along with CRP) were demonstrated to improve the fit of a multivariable risk prediction model for the diagnosis of pneumonia, and other SBIs.

6.2.2. Risk prediction of SBI in the children's ED

The approach taken by the NICE guideline in combining variables known to be associated with SBI can be formalised in the development of a CPR. Here the explanatory variables exert a greater or lesser influence over the risk prediction depending on the strength of their association with the outcome, and with each other.

Though CPRs are not widely established in clinical practice in the setting of the febrile child, research in this area dates back more than 30 years. The earliest prediction rules were developed in infants less than 3 months of age. In this group, the conventional approach had been to admit and treat all, on the basis that in this high-risk group, a missed diagnosis of SBI may have serious consequences. Investigators therefore sought to identify those within this group at the lowest risk who could be safely observed or discharged. This approach led to the development of clinical scores such as those from Rochester[187] and Philadelphia[237] with the ability to identify low-risk infants, and thus rule out SBI.

The previous chapter illustrated the potential value of the use of multivariable risk prediction for the diagnosis of SBI in children in the ED. A simple and parsimonious

model reliably discriminated between SBI and self-limiting illness by combining a small number of clinical variables with a combination of biomarkers. In the internal validation, the model appeared to overestimate the risk of pneumonia, and underestimate the risk of other SBI. In order to estimate the true value of such a model, a process of external validation should now be undertaken[363].

6.2.3. Translating risk prediction into clinical decision-making

Studies of diagnostic accuracy in childhood SBI have so far failed to significantly impact clinical practice. Establishing a prediction rule to support decision-making in clinical practice is a multi-step process[363]. This process was well described by Reilly *et al* and is summarised in Table 37.

Level of Evidence	Definitions and Standards of Evaluation	Implications for Clinicians
Level 1: Derivation of prediction rule	Identification of predictors using multivariable model; blinded assessment of outcomes	Needs validation and further evaluation before using clinically in patient care
Level 2: Narrow validation of prediction rule	Verification of predictors when tested prospectively in 1 setting; blinded assessment of outcomes	Needs validation in various settings; may use predictions cautiously in patients similar to sample studied
Level 3: Broad validation of prediction rule	Verification of predictive model in varied settings with wide spectrum of patients and physicians	Needs impact analysis; may use predictions with confidence in their accuracy
Level 4: Narrow impact analysis of prediction rule used as a decision rule	Prospective demonstration in 1 setting that use of prediction rule improves physician decisions (quality or cost-effectiveness of patient care)	May use cautiously to inform decisions in settings similar to that studied
Level 5: Broad impact analysis of prediction rule used as decision rule	Prospective demonstration in varied settings that use of prediction rule improves physician decisions for wide spectrum of patients	May use in varied settings with confidence that its use will benefit patient care quality of effectiveness

Table 37: Standards of evidence for developing and evaluating prediction rules. Taken from Reilly et al[363].

The SPICED study offered the opportunity to externally validate previously published CPRs, which may provide supportive evidence for their potential impact in future impact studies. We set out to externally validate CPRs published by Brent, Galetto-Lacour and Nijman[7,9,96].

6.2.4. External validation of published clinical prediction rules

6.2.4.1. Brent *et al*

This study prospectively recruited 1951 children under 15 years to a diagnostic accuracy study over two winter periods between 2000 and 2002[9]. The rate of SBI was low (3.8%). A simple risk score comprising clinical variables was derived in 1600 children and internally validated in the remainder. The composition of the score is presented in Table 38.

Clinical variable		Score
Developmental delay	No delay	0
	Delay	4
Risk factor for infection	None	0
	Present	2
State variation	Eyes open	0
	Eyes close briefly	1
	Falls asleep	2
Temperature	<37.5°C	0
	37.5-38.4°C	1
	≥38.5°C	2
Capillary refill time (s)	<2	0
	≥2	1
Hydration status	Well hydrated	0
	Dry mucous membranes	2
	Reduced skin turgor	4
Respiratory rate	Not tachypnoeic	0
	Tachypnoeic	1
Hypoxia	Not hypoxic (SaO_2 ≥95%)	0
	Mild hypoxia (SaO_2 90% - 95%)	1
	Severe hypoxia (SaO_2 <90%)	2

Table 38: Risk prediction score for the diagnosis of SBI derived by Brent *et al*[9]

The risk score discriminated well between SBI and no SBI (*c* statistic 0.77). At a risk threshold of 5 points, the score achieved a NLR of 0.37 (95% CI 0.31 to 0.45) with a sensitivity of 75% and specificity of 68%.

In order to validate the score in the SPICED dataset, the following clinical variables were modified:

State variation, categorised according to the AVPU scale in our dataset was re-categorised. The category 'Awake'(A) was considered equivalent to the 'Eyes open' category reported by Brent, and 'Unresponsive' (U) was considered equivalent to the 'Falls asleep' category. The categories 'Responds to voice' (V) and 'Responds to pain' (P), were combined and assigned to the intermediate 'Falls asleep briefly' category. The proportions of the new category were similar to those reported in the Brent paper.

Hydration status, categorised as 'none', 'mild', 'moderate' and 'severe' in the SPICED dataset were re-categorised as three categories ('none', 'mild', 'severe'), and equated to the 3 categories of the variable in the Brent score ('Well hydrated', 'Dry mucous membranes', 'Reduced skin turgor').

The frequency of the re-categorised variables, along with their univariable odds ratios for the diagnosis of SBI are shown in Table 41.

6.2.4.2. Galetto-Lacour *et al*

The authors derived, and have since externally validated a simple score in children under 3 years based upon three laboratory tests (CRP, PCT and urinalysis, the 'Lab score', Table 39)[7]. The original study (rate of SBI 27%) estimated a sensitivity of 94%, specificity of 78% at a risk score of 3 for the diagnosis of SBI.

Predictor		Points
PCT (ng/ml)	<0.5	0
	≥0.5	2
	≥2	4
CRP (mg/l)	<40	0
	40-99	2
	≥100	4
Urinalysis	Negative	0
	Positive*	1

Table 39: Variables included in the Lab score[7], with weighting based upon OR in univariable analysis. *Positive urinalysis is test positive for either leucocyte esterase, or urinary nitrite.

6.2.4.3. Nijman *et al*

This study combined two large, prospectively collected datasets from the Netherlands in which to derive risk prediction models for the diagnosis of pneumonia, 'other SBIs' and 'no SBI' in children up to 15 years old[96]. The authors identified candidate predictor variables for the diagnosis of SBI from the published literature, and derived a multivariable model inclusive of each irrespective of their statistical contribution (Table 40). The derived model was then validated in a separate population recruited in the UK.

Variable
Age
Sex
Duration of fever
Temperature
Tachypnoea
Tachycardia
Hypoxia (<94%)
Cap refill (>3s)
Chest wall retractions
Ill appearance
lnCRP

Table 40: Variables included in the clinical prediction model by Nijman[96]

6.2.5. Comparing risk prediction models

The identification of numerous risk prediction models poses the question, which is best? Traditional measures of evaluation of risk prediction models focus on discrimination and calibration. Thus improvement in discrimination as measured by the increase in the *c* statistic (or the AUC) of two models is a traditional measure. This has been quantified by DeLong[365], though has been demonstrated to have limited power to detect differences between nested models, and to detect improvements over already discriminatory baseline models[366].

New measures have been developed in order to quantify improvements in model performance and aid comparison. One such measure estimates improvements in classification. This approach defines risk thresholds of clinical importance, and quantifies how often cases are re-classified appropriately into higher risk categories, and non-cases into lower risk categories. This movement can be quantified as a summary 'net reclassification improvement' (NRI)[277]. The NRI has become a popular way to estimate the incremental improvement of additional markers applied to prediction models, and has particular attraction when associated with clinical scenarios in which risk thresholds are well defined, and associated with clear clinical decisions. Where such thresholds fail to exist, the relevance of reclassification across arbitrary thresholds is less clear. In this case, justification of the risk thresholds chosen ought to be made based upon related clinical situations, or upon the event rate. As an alternative, the category-free NRI considers all changes in predicted risk irrespective of risk thresholds, quantifying the net percentage of subjects with or without events who are correctly assigned a different predicted risk. Thus it requires no assumptions to be made about the clinical relevance of risk thresholds. It is of limited value however in quantifying overall model performance, and fails to indicate the potential impact of decision-making based upon the two models[367].

6.3. Hypothesis

Improvements in the classification of febrile children presenting to the ED can be achieved by the addition of the biomarkers PCT and Resistin to a previously validated clinical risk prediction models by Nijman[96].

6.4. Aims and objectives

I. To externally validate published risk prediction models for the diagnosis of SBI in febrile children presenting to the ED[7,9,96]

II. To re-fit the externally validated models in multinomial regression models for the categorical outcome of 'pneumonia', 'other SBI', and 'no SBI'.

III. To estimate the improvement in classification of a multivariable model derived in the SPICED study (the 'SPICED' model) to that achieved by the use of CRP as recommended by the NICE guideline "Feverish illness in children"[184].

IV. To estimate the improvement in classification achieved by the addition of the biomarkers PCT and Resistin to CRP alone.

V. To estimate the improvement in classification achieved by the addition of the biomarkers PCT and Resistin to the validated multivariable model by Nijman (the 'Nijman' model)[96].

6.5. Methods

The SPICED study has been explained in detail in Chapter 5.

6.5.1. Performance characteristics of CRP

The diagnostic accuracy of CRP alone was estimated by developing first a logistic regression model for the diagnosis of SBI, followed by multinomial regression models for the diagnosis of pneumonia, other SBI, and no SBI. As previously described, CRP was fit as a piecewise linear term with a breakpoint at 30 mg/l. Missing data was imputed 10 times using the MICE algorithm described in Section 5.6.3.3, and regression analysis undertaken on the pooled dataset. Discrimination

of the model was measured by *c* statistic, and calibration of the models estimated by the Hosmer-Lemeshow test, and illustrated using calibration plots of predicted risk versus observed frequency of events.

6.5.2. External validation of previously published models

External validation of the three published clinical prediction rules was undertaken in the multiply imputed SPICED dataset. Proxy variables (whereby clinical variables recorded in each dataset were comparable, but not identical) were identified and categorised appropriately. Initial validation of the Brent score and the Lab score was undertaken maintaining the categorical nature of the data (Table 38 and Table 39), and discrimination of the score evaluated using the *c* statistic. Subsequently, logistic regression and multinomial regression models were fitted to the multiply imputed dataset incorporating the variables of the two scores, and discrimination and calibration once more evaluated.

External validation of the Nijman model was performed using the published coefficients of the multinomial regression analysis, and using the transformations described. This model was then re-fitted to the multiply imputed SPICED dataset and discrimination and calibration measured.

6.5.3. Net reclassification improvement

Classification of subjects was compared using Net Reclassification Improvement (NRI). Three category NRI was performed using the 'rap' function in R[368], with weighted reclassification of more than one risk category[367]. Presently, no risk thresholds are established in clinical practice to guide decision-making in febrile children in the ED. We report the use of NRI with the following risk categories: low (<=5%), intermediate (5-20%), and high (>20%). These thresholds equate roughly to half, and double the event rate for both pneumonia (10%) and other SBI (14%). This approach has more clinical utility than the continuous NRI, and is analogous to the 'traffic light' approach taken by the NICE guidance.

6.6. Results

6.6.1. Performance characteristics of CRP

The diagnostic accuracy of CRP was assessed by fitting regression models for the diagnosis of SBI, and subsequently the categorical diagnosis of pneumonia, other SBI, or no SBI to the multiply imputed dataset.

Discrimination of the CRP model for the diagnosis of SBI was limited (*c* statistic 0.75, 95% CI 0.71 to 0.78). Calibration was good, although the model failed to predict children at low risk of SBI (Figure 26). In multinomial regression model for the categorical diagnosis of pneumonia, other SBI and no SBI, discrimination of both models was limited (*c* statistic 0.72 and 0.75 for the diagnosis of pneumonia and other SBIs respectively, Figure 27).

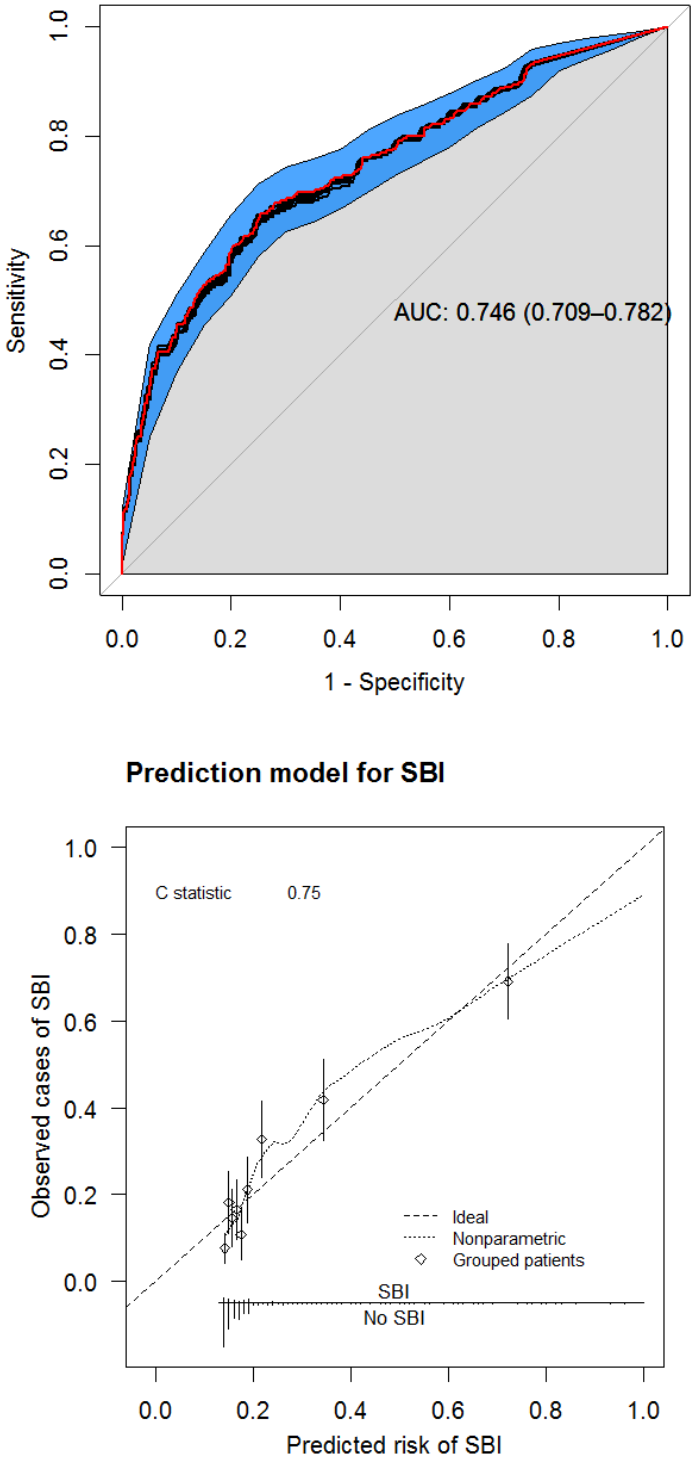


Figure 26: ROC curve (top) and calibration plot (bottom) for the CRP model for the diagnosis of SBI in the multiply imputed dataset. ROC curves are plotted using the linear predictor generated by each of the 10 imputed datasets, and the mean is shown in red. The blue shaded area represents the 95% CI of the ROC curve obtained by 2000 bootstrap replicates.

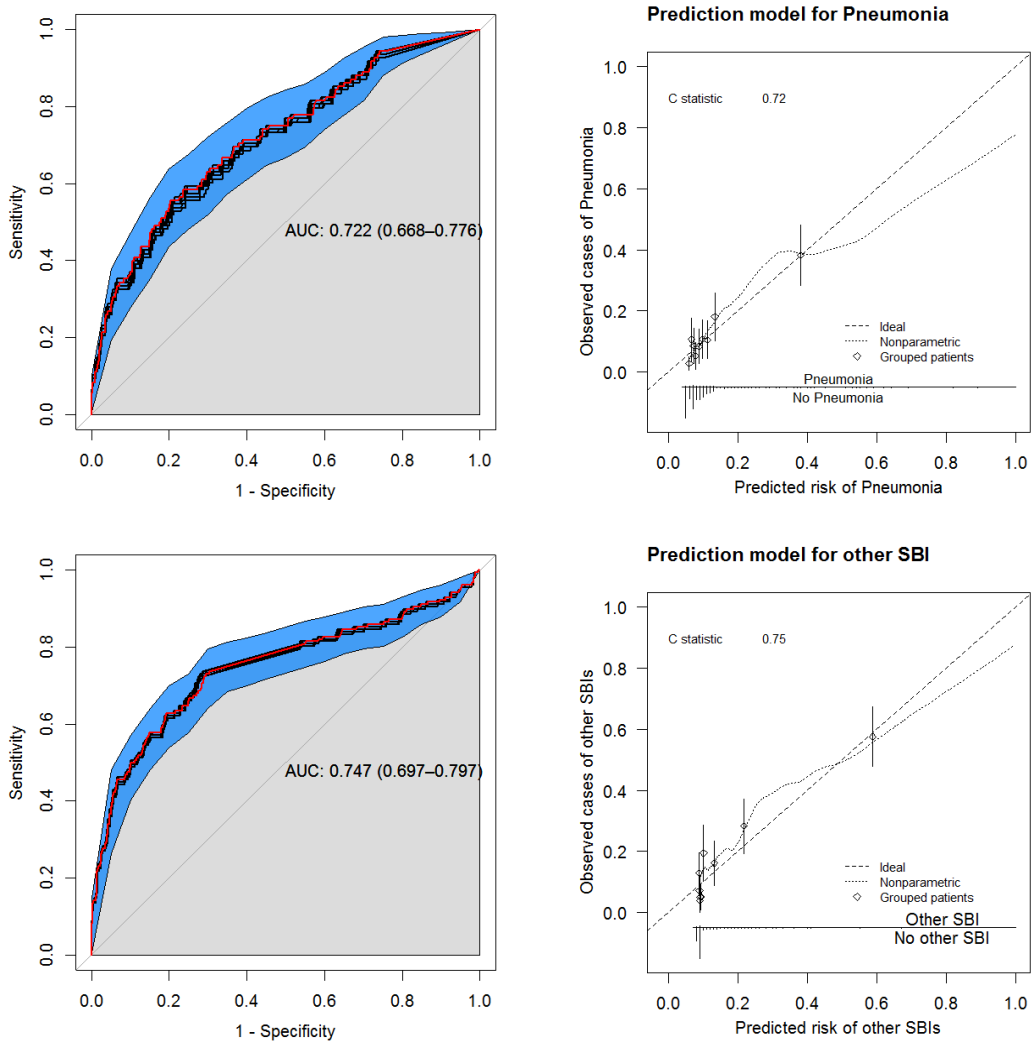


Figure 27: ROC curves (left) and calibration plots (right) of the CRP model for the diagnosis of pneumonia (top) and other SBI (bottom) in the multiply imputed dataset. ROC curves are plotted using the linear predictor generated by each of the 10 imputed datasets, and the mean is shown in red. The blue shaded area represents the 95% CI of the ROC curve obtained by 2000 bootstrap replicates

6.6.2. External validation of previously published risk prediction models

6.6.2.1. Brent clinical risk score

The clinical risk score as described in Table 38 was validated in the multiply imputed SPICED dataset for the binary diagnosis of SBI. The score discriminated poorly between SBI and no SBI with a c statistic of 0.65 (95% CI 0.61 to 0.68, Figure 28).

The variables in the Brent score were the re-fitted to the dataset in a multivariable logistic regression model. Table 41 records the frequency of the categorical variables included in the risk model, and the odds ratios of each variable for the diagnosis of SBI. Discrimination of the model remained poor (*c* statistic 0.65, 95%CI 0.61 to 0.69). Calibration of the model was good, though the calibration plot highlighted that the model was unable to predict children at low risk of SBI (Figure 29).

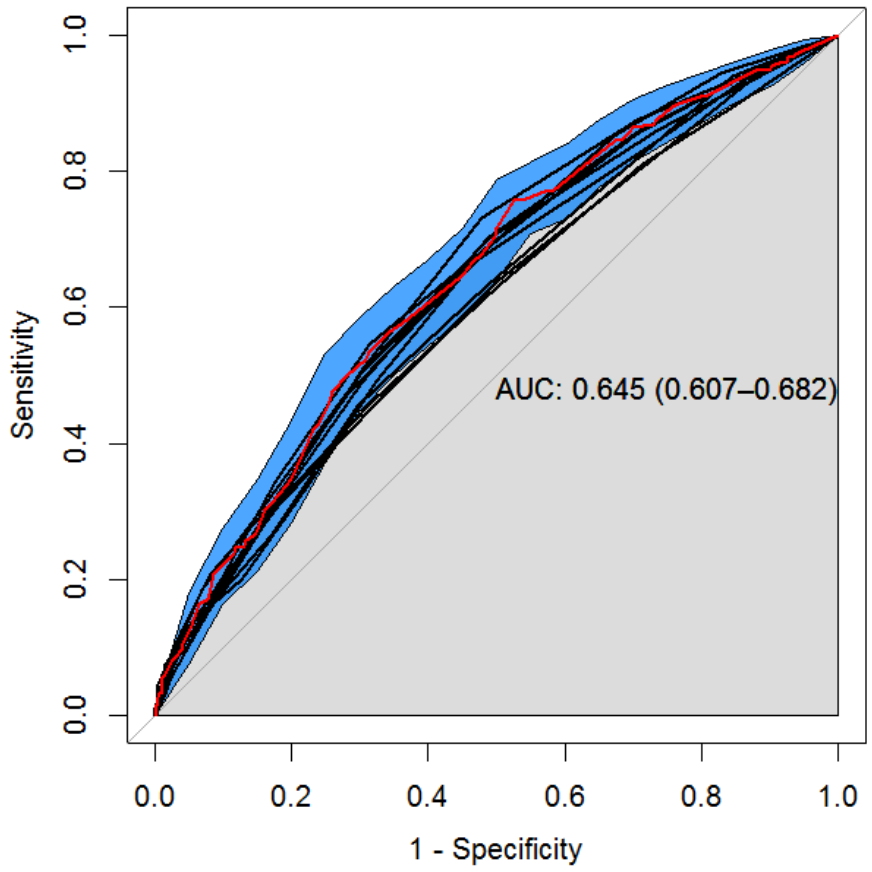
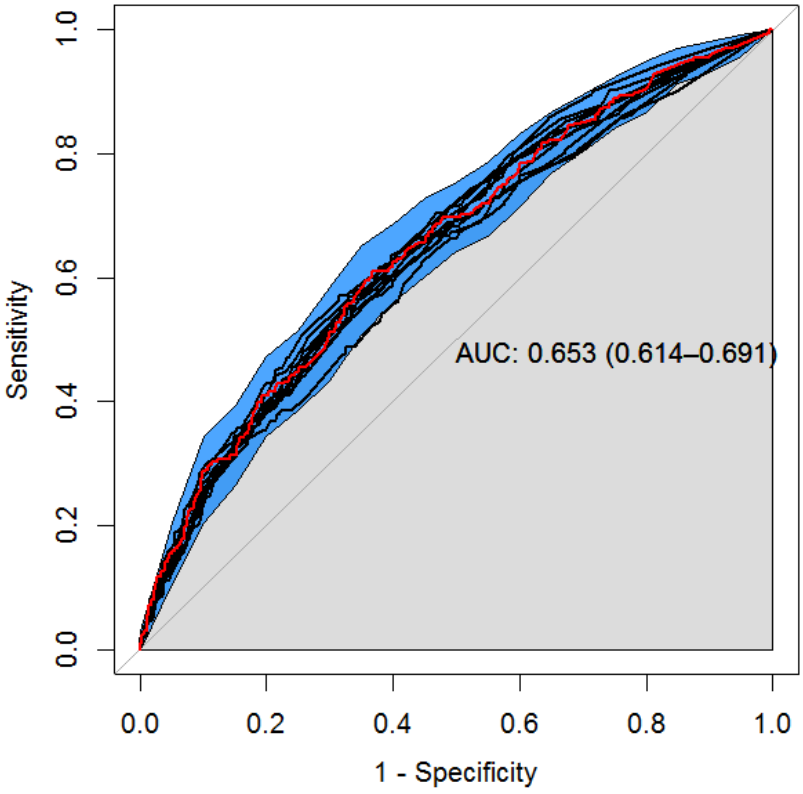


Figure 28: ROC curve for the external validation of the Brent clinical score in the multiply imputed dataset

Variable	Category	n	SBI	%	OR	LCI	UCI
Risk factor for infection	No	1054	244	23.15			
	Yes	43	20	46.51	2.362	1.180	4.727
Developmental delay	No	1043	243	23.30			
	Yes	56	21	37.50	1.335	0.691	2.579
State variation	Awake	1077	257	23.86			
	Briefly asleep	14	3	21.43	0.949	0.251	3.592
	Asleep	5	0	0.00	0.004	0.000	$>1x10^6$
Temp	<37.5	451	95	21.06			
	37.5-38.5	313	75	23.96	1.179	0.812	1.713
	>38.5	337	94	27.89	1.324	0.910	1.927
Tachypnoea	No	654	117	17.89			
	Yes	447	147	32.89	1.804	1.298	2.506
Cap refill	<2s	978	225	23.01			
	>=2s	123	39	31.71	1.186	0.767	1.834
Hypoxia	None (>95%)	904	188	20.80			
	Mild (90-95%)	162	57	35.19	1.572	1.025	2.412
	Severe (<90%)	35	19	54.29	2.162	1.138	4.110
Hydration status	None	355	67	18.87			
	Mild	86	28	32.56	1.336	0.801	2.230
	Severe	16	4	25.00	1.226	0.459	3.271

Table 41: Frequency of categorical variables included in the risk score derived by Brent et al[9], and their odds ratios (OR, with 95% upper and lower confidence intervals) when fit to a multivariable model for the prediction of SBI.



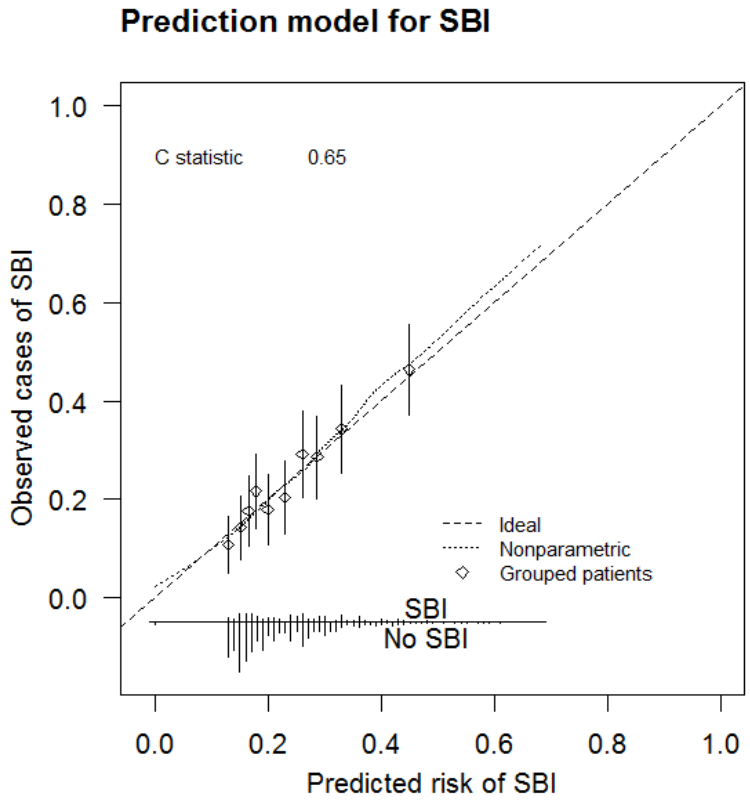


Figure 29: ROC curve (top) and calibration plot (bottom) of the Brent score fitted to the SPICED dataset for the diagnosis of SBI

A multinomial regression model with the categorical outcome 'pneumonia', 'other SBI', and 'no SBI' was then fitted to the data. This approach yielded improved discrimination between pneumonia and no SBI (*c* statistic 0.82), but poor discrimination between other SBIs and no SBI (*c* statistic 0.64,Figure 30). Calibration of both models was good.

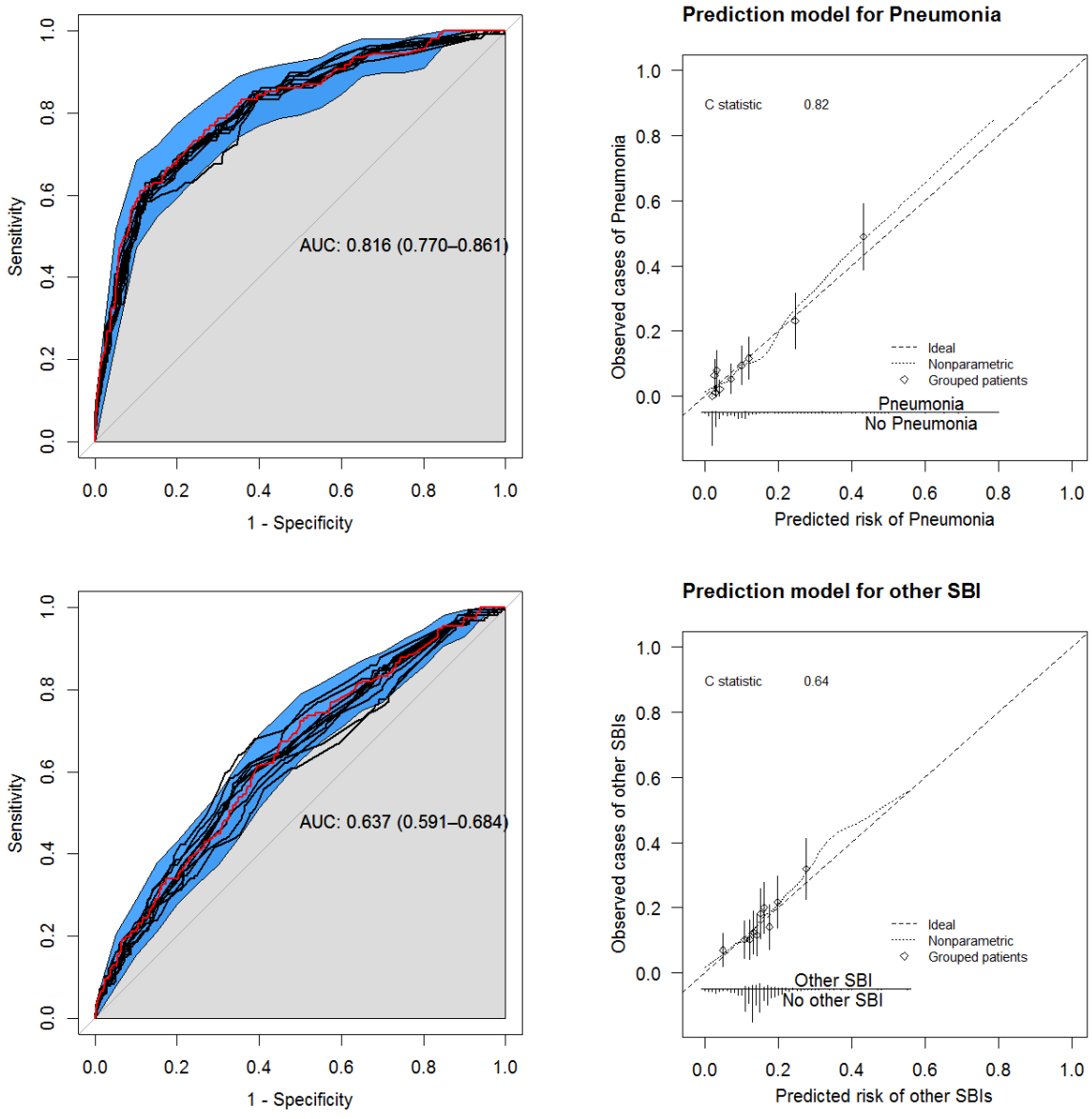


Figure 30: ROC curves (left) and calibration plots (right) for the risk prediction model for pneumonia (top) and other SBI (bottom) based upon the Brent clinical risk score.

6.6.2.2. Galetto-Lacour 'Lab score'

External validation of the Lab score published by Galetto-Lacour et al[7] was performed. The published clinical score (as described in Table 39) demonstrated reasonable discrimination between SBI and no SBI (c statistic 0.79,Figure 31).

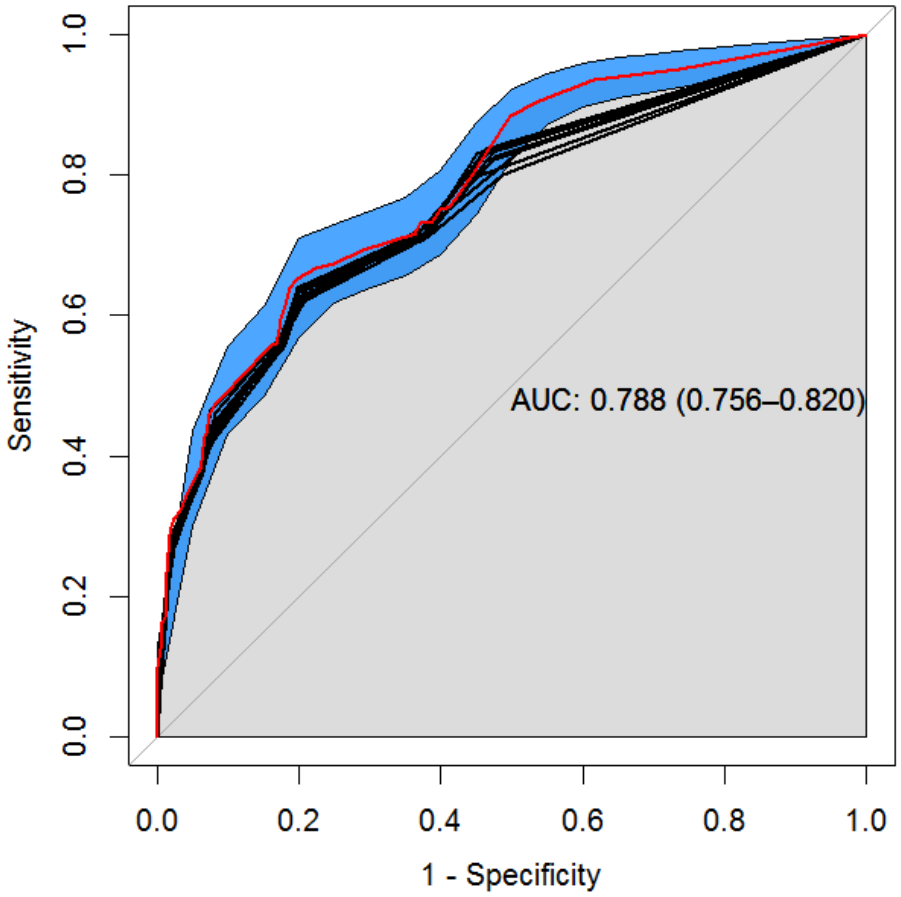


Figure 31: ROC curve of the Lab score performed on the multiply imputed dataset for the diagnosis of SBI.

The variables in the Lab score were fitted to a logistic regression model for the diagnosis of SBI. The *c* statistic estimated by the mean of the linear predictors generated by the multiple imputations was 0.84 (Figure 32). It is notable that the ROC curve generated by the mean of the linear predictors differed significantly from that of the individual imputations, which largely fell outside of the confidence intervals. A complete case analysis was performed in this case (n=223). In this group, the model discriminated well (*c* statistic 0.82, 95% CI 0.74 – 0.90, Figure 33).

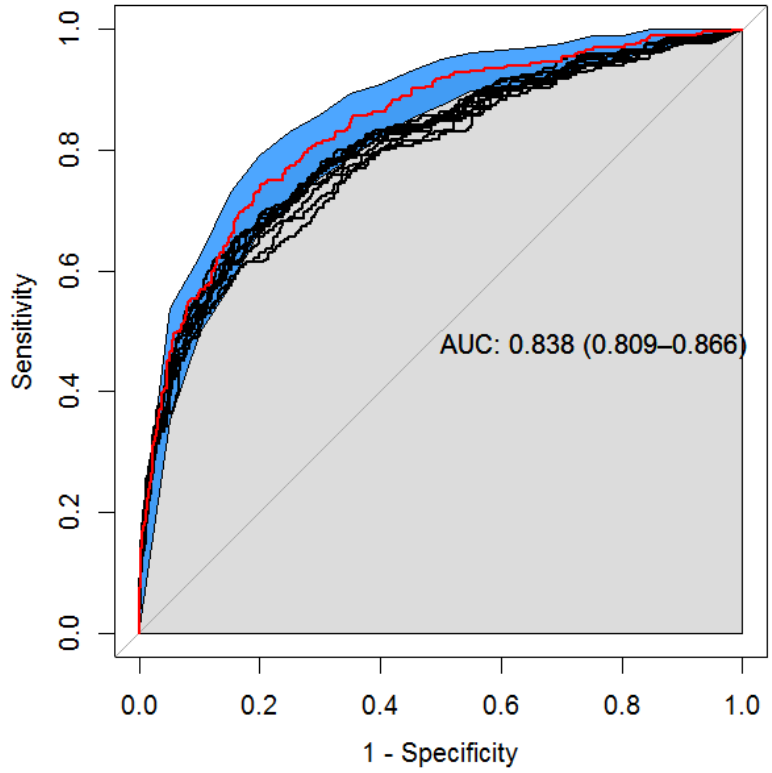


Figure 32: ROC curve of the re-fitted Lab score for the diagnosis of SBI in the multiply imputed dataset.

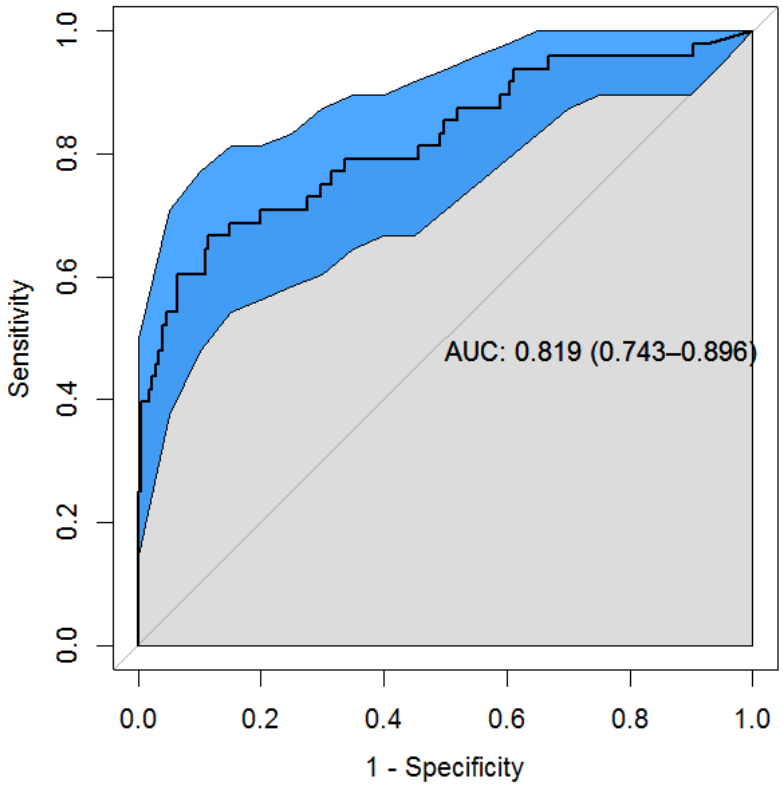


Figure 33: ROC curve of the Lab score for the diagnosis of SBI. Complete case analysis (n=223).

The variables of the Lab score were then fitted to a multinomial regression model, for the categorical diagnosis of pneumonia, other SBI and no SBI (Figure 34).The models discriminated reasonably well, and were well calibrated. For the diagnosis of 'other SBIs', the overall *c* statistic estimated by the mean of the linear predictors was again higher than that of the individual imputed datasets.

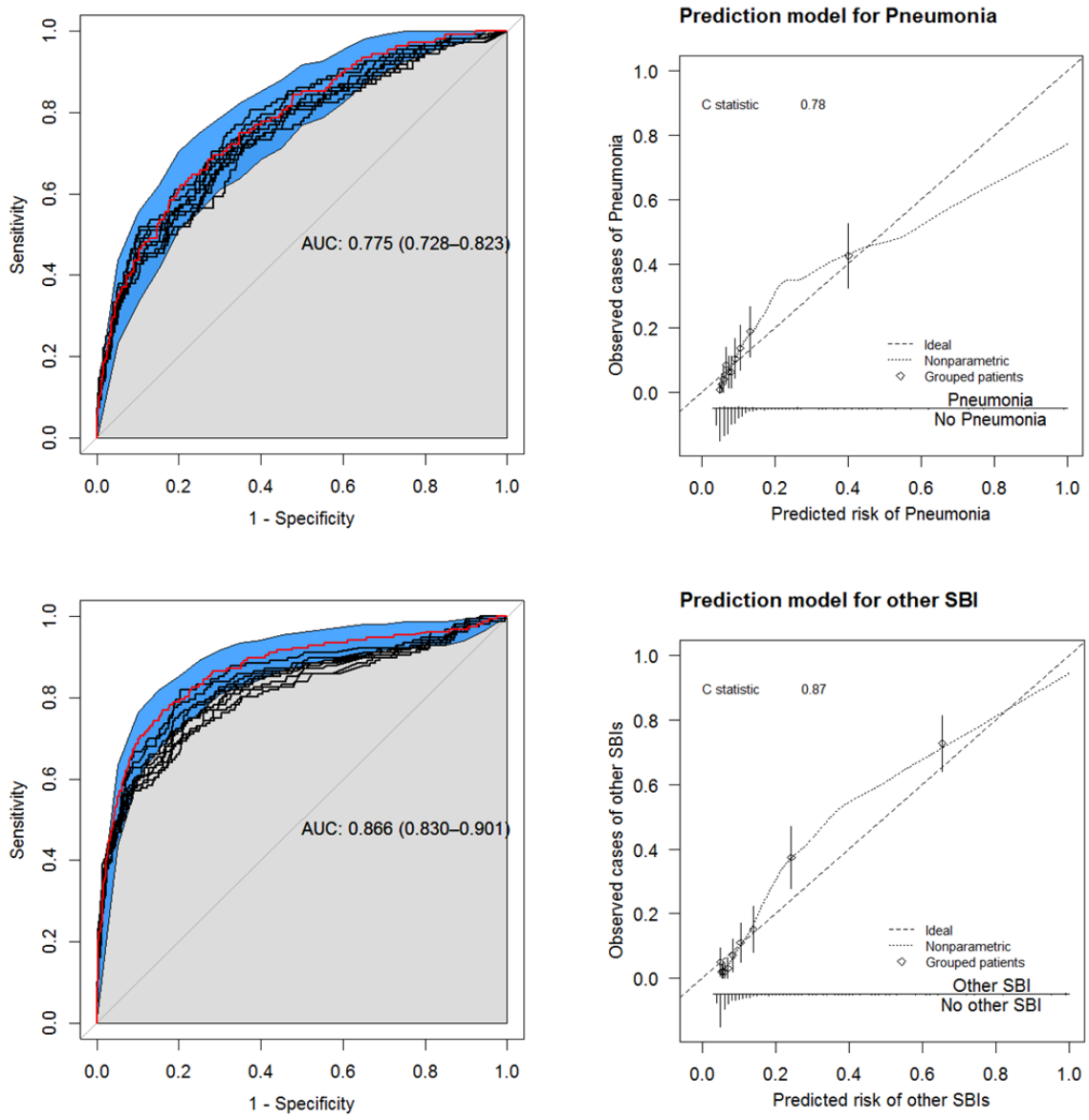


Figure 34: ROC curves (top) and calibration plots (right) for the fitted Lab score model for the diagnosis of pneumonia (top) and other SBIs (bottom)

6.6.2.3. Nijman clinical prediction model

The Nijman model[96] was validated using the published model coefficients. The model discriminated well between pneumonia and no SBI, though performed less well for the diagnosis of other SBIs. The calibration plots suggested an underestimation of the risk of pneumonia, and an overestimation of the risk of other SBIs (Figure 35).

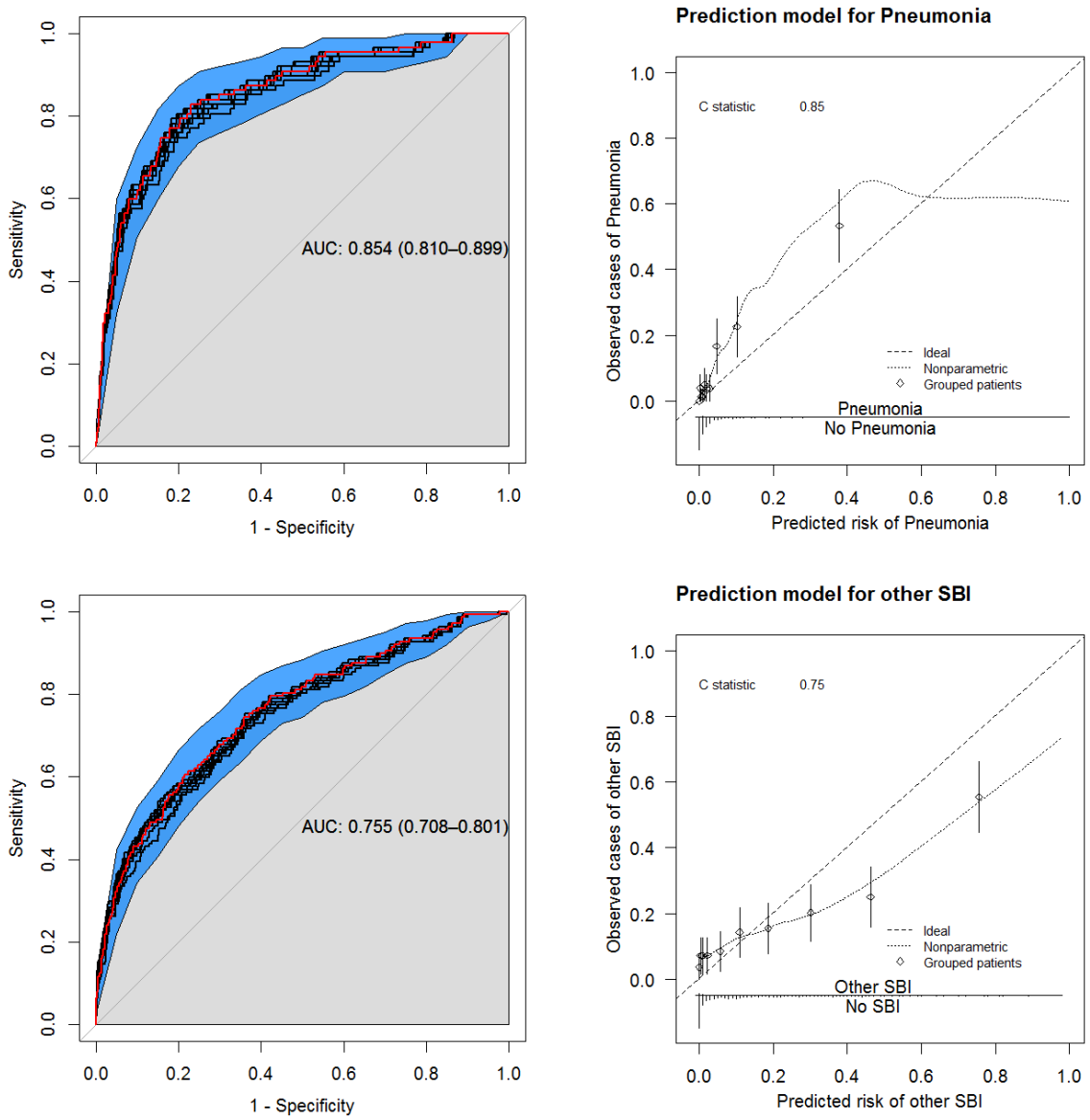


Figure 35: ROC curves (left) and calibration plots (right) of the published multinomial model of Nijman *et al* for the diagnosis of pneumonia (top) and other SBIs (bottom).

The variables in the Nijman model were then re-fitted to the dataset. Model discrimination and calibration was good (Figure 36). The performance characteristics of this re-fitted model at different risk thresholds are recorded in Table 42.

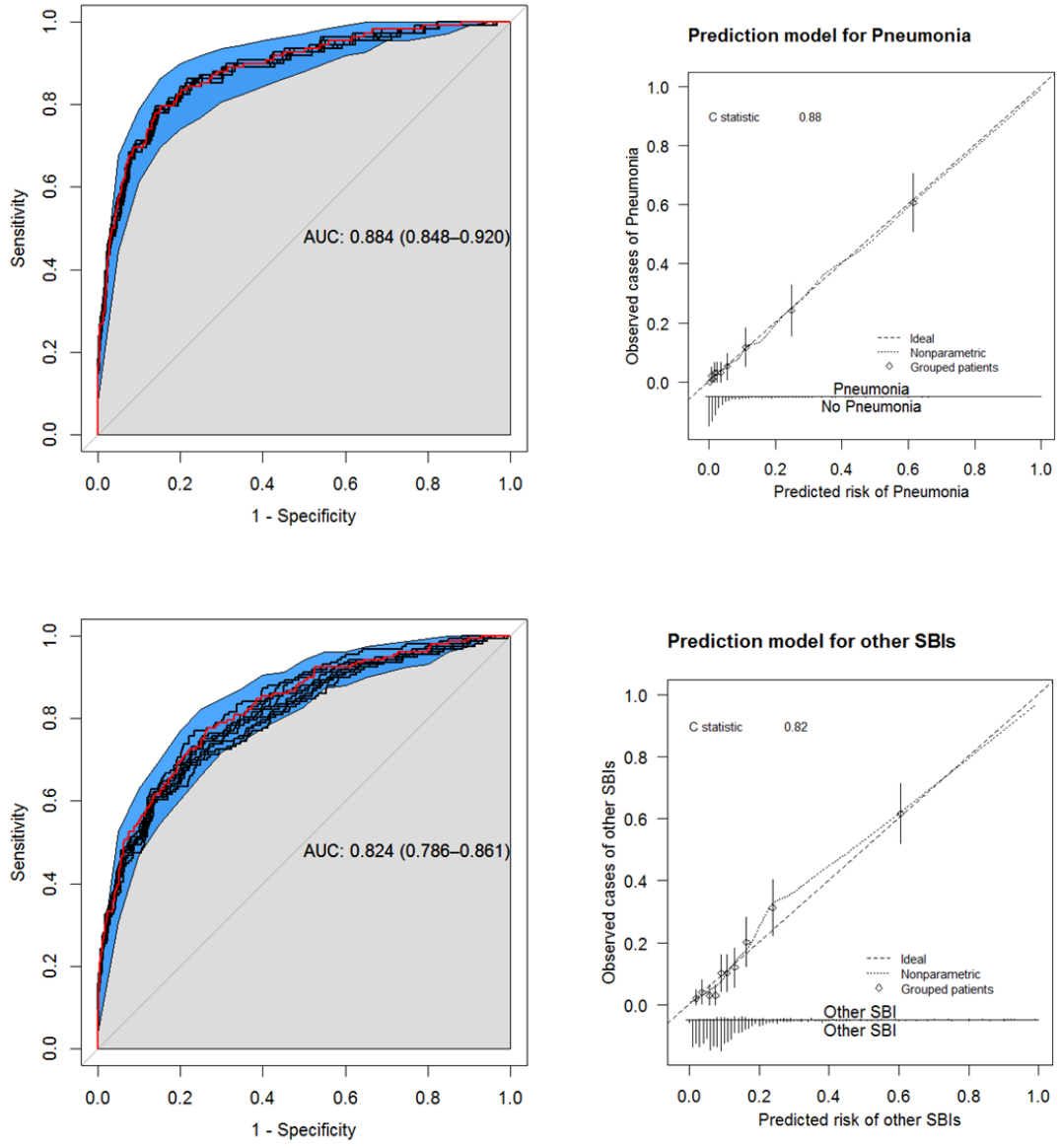


Figure 36: ROC curves (left) and calibration plots (right) of the re-fitted model of Nijman *et al* for the diagnosis of pneumonia (top) and other SBIs (bottom).

Pneumonia												
	Sensitivity	95% CI	Specificity	95% CI	PPV	95% CI	NPV	95% CI	PLR	95% CI	NLR	95% CI
2.5%	0.93	(0.86 - 0.97)	0.51	(0.47 - 0.55)	0.20	(0.16 - 0.24)	0.98	(0.96 - 0.99)	1.91	(1.75 - 2.08)	0.14	(0.07 - 0.28)
5%	0.89	(0.81 - 0.94)	0.70	(0.67 - 0.73)	0.28	(0.23 - 0.33)	0.98	(0.97 - 0.99)	2.98	(2.63 - 3.37)	0.16	(0.09 - 0.27)
10%	0.81	(0.79 - 0.88)	0.82	(0.79 - 0.84)	0.36	(0.30 - 0.43)	0.97	(0.95 - 0.98)	4.41	(3.72 - 5.23)	0.24	(0.16 - 0.35)
20%	0.69	(0.60 - 0.78)	0.89	(0.87 - 0.91)	0.45	(0.38 - 0.53)	0.96	(0.94 - 0.97)	6.46	(5.12 - 8.14)	0.34	(0.26 - 0.46)
30%	0.60	(0.92 - 0.96)	0.94	(0.92 - 0.96)	0.58	(0.48 - 0.67)	0.95	(0.93 - 0.96)	10.5	(7.66 - 14.4)	0.42	(0.33 - 0.53)
Other SBI												
	Sensitivity	95% CI	Specificity	95% CI	PPV	95% CI	NPV	95% CI	PLR	95% CI	NLR	95% CI
2.5%	0.99	(0.96 - 1.0)	0.09	(0.07 - 0.11)	0.17	(0.15 - 0.19)	0.99	(0.93 - 1.00)	1.09	(1.06 - 1.12)	0.07	(0.01 - 0.50)
5%	0.97	(0.93 - 0.99)	0.24	(0.21 - 0.27)	0.19	(0.17 - 0.22)	0.98	(0.95 - 0.99)	1.28	(1.22 - 1.34)	0.13	(0.06 - 0.31)
10%	0.83	(0.77 - 0.89)	0.58	(0.55 - 0.62)	0.27	(0.23 - 0.31)	0.95	(0.93 - 0.97)	1.99	(1.79 - 2.21)	0.29	(0.20 - 0.41)
20%	0.56	(0.48 - 0.64)	0.89	(0.87 - 0.91)	0.49	(0.41 - 0.56)	0.92	(0.90 - 0.93)	5.13	(4.04 - 6.50)	0.49	(0.41 - 0.59)
30%	0.4	(0.32 - 0.48)	0.95	(0.94 - 0.97)	0.61	(0.51 - 0.70)	0.89	(0.87 - 0.91)	8.31	(5.80 - 11.9)	0.63	(0.56 - 0.72)

Table 42: Performance characteristics of the Nijman variables fitted to the multiply imputed SPICED dataset. PPV: positive predictive value, NPV: negative predictive value, PLR: positive likelihood ratio, NLR: negative likelihood ratio.

6.6.3. Net reclassification improvement

6.6.3.1. Comparison of CRP alone with the multivariable SPICED model

Diagnostic accuracy achieved by the use of CRP alone was compared to that achieved by the multivariable SPICED model (Chapter 5). This comparison was undertaken in the validation group. In this group, CRP demonstrated limited discrimination. Discrimination using the *c* statistic was 0.72 (95% CI 0.63 to 0.80) for the diagnosis of pneumonia and 0.75 (0.68 to 0.81) for the diagnosis of other SBIs. Discrimination achieved by the SPICED model was 0.84 (0.78 to 0.90, p=0.004 for the difference) for pneumonia, and 0.77 (0.71 to 0.83, p=0.38) for other SBIs. Reclassification of subjects by the two models is presented in Table 43. There was a substantial improvement in the classification of non-events of 58.9% (50.4 to 67.5%), and 40.4% (33.9 to 46.8%) for pneumonia and other SBI respectively. Though there was no significant change in the classification of pneumonia events, there was a significant reduction in appropriate classification of other SBI events. Of 88 other SBIs in this validation group, 38 were inappropriately classified as low-risk (<5%) by the SPICED model. As the CRP model failed to classify any children as low-risk, this resulted in a NRI of -60.2% (-77.7 to -42.8%). This is consistent with the calibration plots which revealed a higher observed rate of other SBIs than that predicted by the SPICED model (NLR at a threshold of 5% 0.28, 95%CI 0.15 to 0.51, Chapter 5, Table 36).

6.6.3.2. Comparison of CRP with CRP, PCT and Resistin

Diagnostic accuracy achieved by the use of CRP alone was compared to that achieved by the combination of CRP, PCT and Resistin. Discrimination of the multiple biomarker model using the *c* statistic was 0.75 (95% CI 0.70 to 0.80) and 0.78 (95% CI 0.73 to 0.83) for the diagnosis of pneumonia and other SBIs respectively. This compares with values of 0.72 and 0.75 for the CRP model (p-value for the difference 0.04 and 0.06 respectively).

The models were compared using 3 category NRI. Reclassification according to outcome is summarised in Table 44. For the diagnosis of pneumonia, NRI for events

was 3.7% (95% CI -3.1 to 10.5%) and for non-events, NRI was 7.8% (5.6% to 9.9%), and overall NRI 0.11 (0.04 to 0.18). For the diagnosis of other SBIs, there was a 9.6% improvement in classification of events (4.4% to 14.8%), though no difference in the classification of non-events (NRI 0%, -1.5% to 1.5%), and overall NRI 0.10 (0.05 to 0.15). Notably, neither of these models was able to classify children into a low (<5%) risk group.

6.6.3.3. Addition of PCT and Resistin to the Nijman model

The externally validated Nijman model, with variables fitted to the SPICED dataset was compared with an extended model including PCT and Resistin. Discrimination and calibration of the original model (as shown previously in Figure 36) was good. Extension of the model by the addition of PCT and Resistin resulted in an improvement in AUC for the diagnosis of pneumonia from 0.88 to 0.90 (p=0.03) and for the diagnosis of other SBIs from 0.82 to 0.84 (p=0.03)

The performance characteristics of the extended model at various risk thresholds is summarised in

Table **45**. At a low-risk threshold of 5%, the extended model had a sensitivity of 91% (95%CI 84 to 95%) for the diagnosis of pneumonia with a negative likelihood ratio (NLR) of 0.13 (0.07 to 0.24). For other SBIs, the model sensitivity was 97% (93 to 99%), with a NLR 0.12 (0.05 to 0.28). At the high-risk threshold (>20%), the model specificity was 91% (95%CI 88 to 92%) for the diagnosis of pneumonia, with a positive likelihood ratio (PLR) of 7.55 (5.93 to 9.62), and was 91% (88 to 92%) for the diagnosis of other SBI, with a PLR of 6.52 (5.11 to 8.32).

Three category NRI revealed moderate improvements with the extended model (Table 46). For events the NRI achieved by the addition of PCT and Resistin was 2.8% (95% CI -0.4% to 5.9%) and 4.5% (-0.6 to 9.6%) for pneumonia and other SBI respectively. For non-events, this was 1.1% (-0.5 to 2.7%) and 5.3% (3.0 to 7.5%).

Pneumonia=1	Derived multivariable model				NRI				
CRP	<5%	5-20%	>20%	Total	Event	SE	Z	LCI	UCI
<5%	0	0	0	0	0.000	0.104	0.000	-0.204	0.204
5-20%	6	11	12	29					
>20%	1	4	11	16					
Total	7	15	23	45					
Pneumonia=0	**Derived multivariable model**				**NRI**				
CRP	<5%	5-20%	>20%	Total	Non event	SE	Z	LCI	UCI
<5%	0	0	0	0	0.610	0.043	14.059	0.525	0.695
5-20%	294	69	46	409					
>20%	3	12	12	27	Summary NRI 0.61 (0.41 to 0.84)				
Total	297	81	58	436					
Other SBI=1	**Derived multivariable model**				**NRI**				
CRP	<5%	5-20%	>20%	Total	Event	SE	Z	LCI	UCI
<5%	0	0	0	0	-0.602	0.089	-6.786	-0.776	-0.428
5-20%	35	4	7	46					
>20%	3	19	20	42					
Total	38	23	27	88					
Other SBI=0	**Derived multivariable model**				**NRI**				
CRP	<5%	5-20%	>20%	Total	Non event	SE	Z	LCI	UCI
<5%	0	0	0	0	0.851	0.044	19.158	0.764	0.938
5-20%	333	57	2	392					
>20%	9	22	13	44	Summary NRI 0.25 (0.11 to 0.39)				
Total	342	79	15	436					

Table 43: Reclassification of subjects from CRP model to the SPICED model for the prediction of pneumonia (top) and other SBI (bottom) in the validation group

Pneumonia=1	CRP, PCT, Resistin				NRI				
CRP	<5%	5-20%	>20%	Total	Event	SE	Z	LCI	UCI
<5%	0	0	0	0	0.037	0.035	1.069	-0.031	0.105
5-20%	3	64	9	76					
>20%	0	2	30	32					
Total	3	66	39	108					
Pneumonia=0	CRP, PCT, Resistin				NRI				
CRP	<5%	5-20%	>20%	Total	Non event	SE	Z	LCI	UCI
<5%	0	0	0	0	0.078	0.011	7.135	0.056	0.099
5-20%	69	714	9	792					
>20%	0	5	40	45	Summary NRI 0.107 (95% CI 0.038 to 0.176)				
Total	69	719	49	837					
Other SBI=1	CRP, PCT, Resistin				NRI				
CRP	<5%	5-20%	>20%	Total	Event	SE	Z	LCI	UCI
<5%	0	0	0	0	0.096	0.026	3.638	0.044	0.148
5-20%	0	66	16	82					
>20%	0	1	73	74					
Total	0	67	89	156					
Other SBI=0	CRP, PCT, Resistin				NRI				
CRP	<5%	5-20%	>20%	Total	Non event	SE	Z	LCI	UCI
<5%	0	0	0	0	0	0.008	0.000	-0.015	0.015
5-20%	0	737	21	758					
>20%	0	21	58	79	Summary NRI 0.096 (95% CI 0.045 to 0.148)				
Total	0	758	79	837					

Table 44: Reclassification of subjects from CRP model to multiple biomarkers model for the prediction of pneumonia (top) and other SBI (bottom) in the validation group

Pneumonia												
	Sensitivity	95% CI	Specificity	95% CI	PPV	95% CI	NPV	95% CI	PLR	95% CI	NLR	95% CI
2.5%	0.94	(0.88 - 0.98)	0.52	(0.48 - 0.55)	0.20	(0.17 - 0.24)	0.99	(0.97 - 0.99)	1.96	(1.80 - 2.13)	0.11	(0.05 - 0.23)
5%	0.91	(0.84 - 0.95)	0.7	(0.67 - 0.73)	0.28	(0.23 - 0.33)	0.98	(0.97 - 0.99)	3.04	(2.69 - 3.43)	0.13	(0.07 - 0.24)
10%	0.83	(0.75 - 0.90)	0.83	(0.80 - 0.85)	0.38	(0.32 - 0.45)	0.97	(0.96 - 0.98)	4.78	(4.03 - 5.66)	0.20	(0.13 - 0.31)
20%	0.71	(0.62 - 0.80)	0.91	(0.88 - 0.92)	0.49	(0.41 - 0.57)	0.96	(0.94 - 0.97)	7.55	(5.93 - 9.62)	0.32	(0.24 - 0.43)
30%	0.60	(0.50 - 0.69)	0.94	(0.92 - 0.96)	0.57	(0.47 - 0.66)	0.95	(0.93 - 0.96)	10.3	(7.53 - 14.0)	0.42	(0.34 - 0.53)
Other SBI												
	Sensitivity	95% CI	Specificity	95% CI	PPV	95% CI	NPV	95% CI	PLR	95% CI	NLR	95% CI
2.5%	0.99	(0.95 - 1.00)	0.09	(0.07 - 0.11)	0.17	(0.14 - 0.19)	0.97	(0.91 - 1.00)	1.08	(1.05 - 1.11)	0.15	(0.04 - 0.60)
5%	0.97	(0.93 - 0.99)	0.27	(0.24 - 0.31)	0.20	(0.17 - 0.23)	0.98	(0.95 - 0.99)	1.33	(1.27 - 1.40)	0.12	(0.05 - 0.28)
10%	0.83	(0.77 - 0.89)	0.67	(0.63 - 0.70)	0.32	(0.27 - 0.36)	0.96	(0.94 - 0.97)	2.49	(2.21 - 2.80)	0.25	(0.18 - 0.36)
20%	0.62	(0.53 - 0.69)	0.91	(0.88 - 0.92)	0.55	(0.47 - 0.62)	0.93	(0.91 - 0.94)	6.52	(5.11 - 8.32)	0.42	(0.35 - 0.52)
30%	0.50	(0.42 - 0.58)	0.95	(0.93 - 0.96)	0.63	(0.54 - 0.72)	0.91	(0.89 - 0.93)	9.30	(6.72 - 12.9)	0.53	(0.45 - 0.62)

Table 45: Performance characteristics of the Nijman model extended by the inclusion of the biomarkers Procalcitonin and Resistin. PPV: positive predictive value, NPV: negative predictive value, PLR: positive likelihood ratio, NLR: negative likelihood ratio.

Pneumonia=1	Extended Nijman (+ PCT,RTN)				NRI				
Nijman	<=5%	5-20%	>20%	Total	Event	SE	Z	LCI	UCI
<=5%	10	2	0	12	0.028	0.016	1.732	-0.004	0.059
5-20%	0	19	1	20					
>20%	0	0	76	76					
Total	10	21	77	108					
Pneumonia=0	**Extended Nijman (+ PCT,RTN)**				**NRI**				
Nijman	<=5%	5-20%	>20%	Total	Non event	SE	Z	LCI	UCI
<=5%	573	14	1	588	0.010	0.008	1.131	-0.007	0.026
5-20%	14	142	5	161					
>20%	0	15	73	88	Summary NRI 0.039 (95% CI 0.004 to 0.074)				
Total	587	171	79	837					
Other SBI=1	**Extended Nijman (+ PCT,RTN)**				**Modified NRI**				
Nijman	<5%	5-20%	>20%	Total	Event	SE	Z	LCI	UCI
<5%	4	1	0	5	0.045	0.026	1.750	-0.005	0.095
5-20%	1	50	11	62					
>20%	0	4	85	89					
Total	5	55	96	156					
Other SBI=0	**Extended Nijman (+ PCT,RTN)**				**Modified NRI**				
Nijman	<5%	5-20%	>20%	Total	Non event	SE	Z	LCI	UCI
<5%	190	11	0	201	0.053	0.010	5.014	0.032	0.073
5-20%	39	491	11	541					
>20%	0	27	68	95	Summary NRI 0.097 (0.041 to 0.154)				
Total	229	529	79	837					

Table 46: Reclassification of subjects from the Nijman model to a model extended by the inclusion of PCT and Resistin. (Top) pneumonia. (Bottom) Other SBI.

6.7. Discussion

6.7.1. Main results

6.7.1.1. External validation

The SPICED study presented the opportunity to externally validate previously published clinical prediction rules. This external validation is a necessary stage in the process of estimating the true value of a diagnostic test.

Consistent with other evaluations, CRP demonstrated reasonable discrimination between subjects with and without SBI (*c* statistic 0.75), but was unable to identify children at low risk of SBI.

The Brent score failed to discriminate well between subjects with and without SBI (*c* statistic 0.65), nor between other SBI and no SBI (*c* statistic 0.64). The score discriminated better between cases of pneumonia and no SBI (*c* statistic 0.82). This is the first external validation of this risk score. The difference in model performance from that achieved in the derivation study may relate to the significant difference in the rate of SBI (24% v 3.8%).

The Lab score demonstrated reasonable discrimination between SBI and no SBI (*c* statistic 0.79). When the model was re-fitted using CRP and PCT as continuous variables, a discrepancy was evident between the ROC curves plotted by the linear predictors of the individual imputations, and that of the pooled linear predictors. This is likely a result of the high proportion of urinalysis observations missing (80%). A complete case analysis (n=223) estimated a *c* statistic of 0.82 (95% CI 0.74 to 0.90). Using multinomial regression analysis, the model appeared to discriminate better between other SBI and no SBI, than pneumonia and no SBI (*c* statistic 0.87 and 0.78 respectively). Model calibration was acceptable.

External validation of the Nijman model revealed encouraging discrimination (*c* statistic 0.85 and 0.76 for the diagnosis of pneumonia and other SBI respectively). Calibration of the models was good, though the models underestimated the risk of pneumonia and overestimated the risk of other SBIs.

6.7.1.2. Comparison using net reclassification improvement

Comparison of CRP alone with a combination of CRP, PCT and Resistin revealed improvements in classification for both pneumonia and other SBI (overall NRI 0.11 and 0.10 respectively). The addition of the biomarkers PCT and Resistin particularly improved the classification of other SBI events (NRI 9.6%, 95% CI 4.4 to 14.8%), and pneumonia non-events (NRI 7.8%, 5.6% to 9.9%).

There was a substantial improvement in classification when comparing the SPICED model to CRP. Overall NRI was 0.61 (0.41 to 0.84) for pneumonia, and 0.25 (0.11 to 0.39) for other SBIs. In particular this was a reclassification improvement of non-events for both pneumonia (61%, 41 to 84%) and other SBI (85%, 76 to 94%). This reflects the failure of the CRP model to identify children at low risk (<5%). With the SPICED model, however, there was a 60% (43 to 78%) inappropriate net reclassification of other SBIs to lower risk categories. The baseline CRP model has a sensitivity of 100% at this low-risk threshold, as it fails to classify any subject below this level. By contrast at a 5% risk threshold the SPICED model only achieves a sensitivity of 58% and a NLR of 0.54 (see Table 36, Chapter 5). The SPICED model is not sufficiently sensitive to rule out other SBIs. For the diagnosis of pneumonia, at a risk threshold of 5%, the SPICED model achieves a NLR of 0.14, and a sensitivity of 91%.

The addition of the biomarkers PCT and Resistin to the published Nijman model achieved a small improvement in discrimination for both the pneumonia and other SBI models (*c* statistic increased from 0.89 to 0.90, and from 0.82 to 0.84 respectively; p=0.03 for the difference), and in classification. Overall NRI was 0.04 and 0.10 for pneumonia and other SBI models respectively. Significantly, there was a 5.3% (3.2 to 7.3%) improvement in the classification of non-events achieved by the extended other SBI model. This incremental improvement was achieved despite a good baseline model.

6.7.2. Strengths

This external validation presents further evidence of the value of multivariable risk prediction in our large, prospectively recruited sample. In particular the performance characteristics of the Nijman model are encouraging. The application of multiple risk thresholds has the potential to support rational decision-making in the ED. A low-risk threshold (<5%), with a NLR of 0.16 (pneumonia) or 0.13 (other SBIs) may support a decision to discharge, and to withhold antibiotic treatment. Conversely a high-risk threshold (>20%) with PLRs of 6.46 and 5.13 for pneumonia and other SBIs respectively, may expedite prompt recognition and treatment. This approach results in a substantial improvement in classification when compared to the current NICE recommendation of the use of CRP in children considered at risk of SBI. In particular, the risk model allows the reclassification of children into a low-risk category (<5%) which has the potential to support the clinician to rule out SBI. The reliable classification of children into risk categories could be incorporated into decision rules to guide clinicians regarding further investigation, treatment and disposition (admit or discharge).

We have estimated the benefit of the addition of PCT and Resistin to the Nijman model. Using net reclassification improvement, we suggest that this approach may have a role in improving rational decision-making, particularly in the process of ruling out SBI. In the context of a recent increase in hospital admissions for short-stay uncomplicated admissions for young children[22,49], and also with regard to rational antibiotic use at a time of increasing antimicrobial resistance, this deserves further exploration.

We propose the use of 3 category NRI utilising risk thresholds of 5% and 20% to define low, intermediate and high-risk children. This categorisation is not yet established in the context of predicting SBI in the children's ED, but is based upon the event rate in this setting, and is analogous to the approach advocated by the current NICE consensus guideline. Our analysis provides clinically useful indications of the relative value of risk models in this setting, and could form the basis of decision rules upon which to perform impact analysis.

6.7.3. Limitations

The SPICED study was a large, prospective study which collected data on multiple clinical and laboratory variables. Despite significant investment from both research and clinical teams, data recording was not complete. In the busy children's ED, this is not unusual, and substantial efforts were made to mitigate the problem of incomplete data. We have undertaken a process of missing data imputation based on the assumption of the data being missing at random, and have demonstrated plausible missing data imputations. Importantly, assessments of the discrimination of the individual imputed datasets largely fell within the confidence interval of the pooled analysis. In the external validation of the Lab score, however, this appears not to be the case. Given the simple structure of the Lab score, which is based upon only three variables, and the proportion of missing data of one of these variables in our dataset (urinalysis was only recorded in approximately 20% of the SPICED dataset), this is perhaps unsurprising. Our complete case analysis should be interpreted with caution, as the performance of urinalysis is associated with the outcome. That is, the test was performed in those in whom it is most likely of value.

We have undertaken a comparison of a multivariable model with that of CRP alone, on the basis that CRP is advocated in febrile children considered at risk of SBI. We acknowledge that clinicians do not use CRP in isolation, but believe that the study population constitutes the same group identified as being at risk in the NICE guidance, and our assertion is that evaluating risk in an integrated way in this group is preferable to interpreting a blood test performed in addition to the clinical assessment.

6.7.4. Next steps

The demonstration of a discriminatory risk prediction model (the Nijman model) on external validation prompts further research to evaluate the impact of decision-making based upon such a model. This process of impact analysis is important and often overlooked in diagnostic research[363], and particularly so in children[234].

The evaluation of impact requires that discriminatory risk prediction rules or models are developed into practical clinical decision rules (CDRs). CDRs help clinicians to make specific decisions based upon discriminatory and accessible diagnostic variables which are evidence-based[369]. This impact analysis of CDRs falls within the remit of implementation science. Implementation science evaluates the methods which support sustained uptake of research findings into clinical practice. The demonstration of adequate risk prediction may not be sufficient to convince clinicians of the value of this risk based decision-making.

Recent impact studies in the area illustrate this challenge. The Lab score was evaluated in a randomised controlled trial of antibiotic prescribing in 271 children under 3 years with fever without source. Though the Lab score performed well in predicting SBI (AUC 0.91), and recommended 30% fewer antibiotic treatments than the control, no reduction of antibiotic prescribing was demonstrated due to lack of adherence to the decision algorithm[370]. Similarly, a CDR based upon the Nijman risk prediction model also failed to demonstrate impact in a recent randomised controlled trial of 439 children up to 16 years[371]. In this case, risk predictions remained reliable, and compliance with the CDR was high, but no impact upon outcomes such as length of hospital (or ED) stay, or likelihood of hospital admission were observed.

Such findings highlight the need to understand barriers to the implementation of evidence-based CDRs. The involvement of clinicians and families in discussions about how to translate estimates of risk into sound clinical decision-making is necessary. The agreement of appropriate risk thresholds for example will require consensus agreement if they are to underpin decision rules. Whilst there may be a general acceptance that too many febrile children receive unnecessary treatment, any recalibration of risk which increases the possibility of missed SBI will require careful consideration.

6.8. Conclusions

Multivariable risk prediction in febrile children of all ages presenting to the ED is possible. In particular this approach has the potential to safely rule out SBI, and improve decision-making regarding admission to hospital and rational antimicrobial prescribing. Well-designed implementation studies are required to identify barriers to the introduction of CDRs based upon robust and discriminatory risk prediction models.

Chapter 7: Discussion

7.1. Major findings

The work presented in this thesis illustrates the ongoing challenge of the recognition of SBI in febrile children presenting to the ED. We detail how the aetiology of bacteraemia has evolved over the last decade and the implications of this in terms of recognition and appropriate antimicrobial therapy. We demonstrate the use of 16S rRNA PCR with sequencing (SepsiTest) in whole blood for the first time in this setting, and conclude that at present this approach remains only an adjunct to blood culture in high-risk children. Finally we present further evidence of the ability of multivariable risk prediction to discriminate between children with SBI and those without.

7.1.1. Bacteraemia in the children's ED is increasingly healthcare-associated

Our data describe changes in the aetiology of bacteraemia between 2001 and 2011 in children presenting to the ED. Recent national studies in the UK have highlighted a reduction in the incidence of vaccine-preventable bacteraemia, and an increase in Gram negative bacteraemia in children[12,40]. This work illustrates that these trends are true of children presenting to the ED. We estimate that the introduction of the PCV was associated with a reduction in pneumococcal bacteraemia of approximately one half. Infections previously considered 'community-acquired' are increasingly 'healthcare-associated' (HCA), with implications for both recognition and prompt, effective antimicrobial therapy. An increasing proportion of children with serious infections have significant comorbidities[372]. These HCA infections required a longer inpatient stay which is an important, if not unexpected, observation.

Though time to empirical antibiotics was not clearly shown to increase in HCA bacteraemia, time to antibiotics was significantly longer in Gram negative infections (and indeed all other groups) than in vaccine-preventable infections. These infections are increasing in frequency, and make up a greater proportion of bacteraemia in the ED than they previously did. Even accounting for changes in the aetiology, time to antibiotics increased from 2001 to 2011.

The increasing frequency of HCA bacteraemia in the children's ED (from 0.17 to 0.43 per 1000 presentations) emphasises the importance of developing appropriate empirical antimicrobial protocols. Over the course of the years studied, the likelihood that an isolated organism was susceptible to the empirical antibiotic choice declined. Though we did not demonstrate adverse outcomes (such as death, or admission to PICU) associated with ineffective empirical therapy, adult studies of bacteraemia have identified an increase in mortality associated with inadequate empirical antibiotics[282,283]. As these outcomes are relatively rare in children, it may be that our cohort was simply too small to detect a difference.

7.1.2. Broad-range molecular identification (SepsiTest) in whole blood is possible in children presenting to the ED, and augments the use of blood culture

Difficulty in recognising bacteraemia coupled with changes in the likely aetiology, and a growing likelihood of non-susceptibility to empirical therapy all highlight the need for improved diagnostics for bacteraemia. To date, despite the limitations of blood culture, no culture independent method of identification has been shown to significantly improve the diagnosis of bacteraemia in children.

Our evaluation of SepsiTest, 16S rRNA PCR with sequencing is the first to be undertaken in febrile children presenting to the ED. Using clinical samples of 1ml of whole blood we have demonstrated that this approach is possible. The sensitivity of SepsiTest is comparable to that of blood culture, though the higher rate of identification by SepsiTest of organisms of questionable significance resulted in a reduced specificity. Both failed to identify important pathogens including 3 cases of meningococcal septicaemia diagnosed by a *N.meningitidis* specific PCR. Their combined use may improve the sensitivity of the diagnosis of bacteraemia in the children's ED.

The moderate improvement in yield, and the potential of more rapid pathogen identification offered by SepsiTest needs to be weighed against the added costs of the approach. Not only would the use of SepsiTest incur its own cost (estimated at approximately £140 per sample in a 2009 paper[83]), but the implications of a high

rate of identification of possible bacteraemia include prolonged length of stay and antimicrobial use. In our sample, we identified 41 false positive results (in comparison with 6 blood culture isolates which were considered 'contaminants'). Some of these – and we highlight the common finding of Viridans Group Streptococcus – may reflect true, possibly transient, bacteraemia, but it is not clear that their identification would have improved the clinical management of these children. Most were admitted to hospital for significantly shorter than might be expected of a child with proven bacteraemia, and many received only a short course of antibiotics, including some who received no intravenous antibiotics at all.

Stratifying risk using biomarkers, or clinical risk prediction models, may help to identify children in whom the use of SepsiTest is most of value. This approach could balance the benefits of identifying culture negative infections, as well as potentially expediting the diagnosis, against that of the increased false positive rate. A Health Technology Assessment-funded cost-effectiveness analysis of SepsiTest alongside SeptiFast and Iridica (the most recent iteration of the PLEX-ID MS ESI platform, see Section 4.1.2.2) is currently ongoing (http://www.crd.york.ac.uk/PROSPEROFILES/16724_PROTOCOL_20150112.pdf).

7.1.3. Multivariable risk prediction discriminates between children with SBI and those with self-limiting infections

This large, prospective sample of febrile children of all ages attending the ED allowed us to derive multivariable risk prediction models for the diagnosis of pneumonia, 'other SBI' and no SBI. On internal validation, a parsimonious multivariable risk prediction model combining clinical features (respiratory rate, and 'normal air entry') and the biomarkers CRP, PCT and Resistin discriminated well between pneumonia and no SBI (*c* statistic 0.84, 95% CI 0.78 to 0.90) and between 'other SBI' and no SBI (*c* statistic 0.77, 95% CI 0.71 to 0.83). Importantly, we also performed external validation of a previously published model (Nijman *et al*[96])which confirmed the value of this model in our new sample (*c* statistic 0.85, 95% CI 0.81 to 0.90 for pneumonia, and 0.76, 95% CI 0.71 to 0.80 for 'other SBI'). Two further external validations of published CPRs (by Brent, and Galetto-Lacour) were undertaken. The Brent score discriminated poorly between SBI and no SBI (*c*

statistics 0.65), while the Galetto-Lacour 'Lab score' performed better (*c* statistic 0.79 for the diagnosis of SBI). The evaluation of the Lab score was however limited by the use of urinalysis (one of three variables in the Lab score) in only 20% of the SPICED dataset.

The use of risk prediction models allowed the estimation of performance characteristics at various risk thresholds. On the basis of this evidence, risk thresholds may be established upon which to propose clinical decisions. For example, using the derived multivariable model, at a risk threshold of 20%, the specificity of the model for the diagnosis of pneumonia was 84% (sensitivity 62%), with a PLR of 3.82 (95% CI 2.8 to 5.2). For 'other SBI', the equivalent values were 88% (sensitivity 63%), with a PLR 5.14 (95% CI 3.8 to 6.9). These values would support a 'rule in' decision and guide appropriate treatment.

The performance characteristics of the externally validated 'Nijman' model were encouraging. At the 20% risk threshold suggested above, the specificity of the model for the diagnosis of pneumonia was 89% (sensitivity 69%), with a PLR of 6.5 (95% CI 5.1 to 8.1). For 'other SBI' the values were 89% (sensitivity 56%) with a PLR of 5.1 (95% CI 4.0 to 6.5). At a low-risk threshold of 5%, the pneumonia model achieved a sensitivity was 89% (specificity 70%) with a NLR of 0.16 (95% CI 0.09 to 0.27), while the 'other SBI' model achieved equivalent values of 97% (specificity 24%) with a NLR of 0.13 (95% CI 0.06 to 0.31). The application of these risk thresholds to guide treatment decisions would appear to reliably predict a low-risk group in whom SBI can be ruled out, and a high-risk group in whom treatment ought to be initiated. There remains an intermediate group for whom further observation or investigation would be necessary, and importantly there remains residual misclassification. This may be particularly important in the context of ruling out SBI, and emphasises the ongoing importance of safety netting[48].

Comparison of the performance of the risk prediction models was made by estimating improvements in classification. The first comparison was with a model based upon CRP, as CRP is currently recommended by the NICE 'feverish illness in children' guideline to be used in children considered at risk of SBI. Using 3-category

Net reclassification improvement (NRI) to compare the derived multivariable model with CRP alone, the derived model improved the classification of non-events (by 61% for pneumonia, and 85% for 'other SBI'), but this was achieved at a cost of wrongly reclassifying 60% of 'other SBI' events into a lower risk category than that determined by the CRP model. NRI was also used to estimate the improvement in classification obtained by the addition of the biomarkers PCT and Resistin (which were found to be of value in the derived multivariable model) to the published model by Nijman *et al*. This comparison yielded modest improvements in classification, in particular a 5.3% (95% CI 3.2 to 7.3%) improvement in reclassification of 'other SBI' non-events.

7.2. Comparison with other studies

Our findings are consistent with national data in describing a decline in vaccine-preventable bacteraemia, and an increase in Gram negative bacteraemia[12,40]. Our estimate of a 49% reduction in pneumococcal bacteraemia attributable to the introduction of the PCV is comparable with other similar estimates of reductions in invasive pneumococcal disease[20,287,288]. The rate of bacteraemia observed (1.4 per 1000 attendances) is lower than that of a study of 15781 febrile children attending ED in Australia (4.1 per 1000)[6]. The starting point of our study was the identification of all positive blood cultures in children who presented via the ED. This is inclusive of all children, irrespective of presentation, and so includes children who presented with features other than fever, and to non-medical specialities (such as general, orthopaedic and ENT surgeons). The denominator used then was all non-trauma presentations, rather than acute febrile illnesses which helps to explain this variation.

We observed an increase in the rate of HCA bacteraemia, as defined in the study, from 0.18 to 0.50 per 1000 ED attendances (p=0.002), and a reduction in CA bacteraemia (from 1.18 to 0.71 per 1000 ED attendances, p=0.005). The proportion of bacteraemia that was HCA increased from 13% to 41%. HCA bacteraemia was associated with an increased LOS. This has previously been observed in adults[291],

though not children. We based the definition of HCA bacteraemia upon markers of frequent exposure to the healthcare environment and with reference to a recent published systematic review of the subject[281]. This review summarised the criteria used to distinguish CA, HCA and hospital-acquired bacteraemia, as well as the relative proportions of each. It identified little evidence upon which to estimate the proportion of bacteraemia isolates considered CA, HCA and hospital-acquired in children. When neonates were excluded, the mean proportion of episodes which were CA was 58% (95% CI 55-61%), and in the one study to report all 3 categories of infection, HCA was estimated to account for 26% of bacteraemia episodes[373]. There were no data exclusively from the paediatric ED. It concluded that to date inconsistent criteria had been applied to studies of bacteraemia in children and that population-based studies which incorporate clinical as well as microbiological data should be undertaken in order to define clear evidence-based criteria for each category of infection. Such an approach, it was argued, may support the clinician to determine the likeliest organism and most appropriate empirical therapy.

The use of SepsiTest has not previously been described in children. To date, evaluations of 16S rRNA PCR as a broad-range molecular test for bacteraemia have been limited to the NICU, and the results have been variable. One study reported a 100% sensitivity for the diagnosis of 15 cases of 'sepsis and meningitis' (of 190 neonates sampled) when compared with culture alone[342], though a second study of 295 neonates reported a sensitivity of only 42% when compared with blood culture[341]. In a recent ED evaluation of SepsiTest and Magicplex (a multiplex real-time PCR) in 125 adults, SepsiTest was reported to have a sensitivity of only 11% (3/27) compared with blood culture[344]. Consistent with this, studies of alternative platforms have produced conflicting results. This is evidenced most clearly by the recent meta-analysis of SeptiFast which estimated summary sensitivity and specificity (compared with blood culture) as 68% (95% CI 63 to 73%) and 86% (95% CI 84 to 89%) respectively. It concluded however that methodological weaknesses in the studies rendered the analysis unreliable[80].

We identified a number of clinical signs of SBI broadly consistent with other published studies[10]. Features of an abnormal respiratory examination (increased

work of breathing, abnormal auscultation, and hypoxia) all significantly increased the likelihood of pneumonia in multinomial analysis, while neck stiffness, a bulging fontanelle and irritability were associated with the presence of 'other SBIs'. We failed to demonstrate an association with other general features of the clinical assessment such as an 'ill appearance', or parental concern. In each of these cases, we had a significant problem of missing data. Our data suggest that a petechial rash (commonly observed in the study owing to the NICE guidance which advocates laboratory testing in all febrile children with a petechial rash) only moderately increased the likelihood of bacteraemia (PLR 1.52, 95% CI 0.92 to 2.50). Whilst a number of studies have supported its use as a 'red flag' sign of serious infection[10], the majority of these were undertaken specifically in children suspected of meningitis[91,98], or MCD, in which the proportion of children with proven MCD was between 10 and 15%[94,95]. Such estimates of the risk of MCD or meningitis are unrepresentative of the risk in febrile children presenting to the ED. A more recent evaluation of petechial spots in the context of the NICE guideline provided less compelling evidence of its value as a 'red flag'. Of four datasets which included data on 'non-blanching rash', two estimated PLRs >5[98,374], though one of these selected children on the basis of fever and meningeal signs[98]. Two further studies of unselected febrile children (under 5 years) estimated PLRs of 1.7 (95% CI 0.9 to 3.4)[249] and 0.4 (95% CI 0.1 to 2.8)[375] respectively. We draw a distinction between petechial rash and purpura, which occurred rarely in our study (n=10), and which is likely to be of more clinical significance. While petechial rashes may indicate serious illness, including invasive infection, we question its continued inclusion as a 'red flag' feature in the NICE guidance.

Recent diagnostic accuracy studies have demonstrated the value of multivariable risk prediction in the diagnosis of SBI in children in the ED. Whilst we approached the derivation of a risk prediction model using a stepwise model building approach, both Nijman *et al*[96] and Craig *et al*[6] pre-determined the variables to be included in their risk models. This was done on the basis of a substantive understanding of the importance of these variables in clinical practice. Model selection based upon stepwise methods is commonly used, but has been criticised for underestimating

the confidence intervals of the parameter estimates, and of ignoring the effects of variables with small, but potentially interesting effects[362]. In order to mitigate this, we included a validation sample, in which the derived model performed reasonably well.

The external validity of studies such as this depends upon the population selected. We included children up to 16 years (that is, the great majority of children presenting to the ED), and did not select on the basis of clinical presentation (other than 'history of fever'). We included children with comorbidities, with the exception of immunodeficiency, and children who had received prior antibiotics as we considered that these children represented an important risk group for SBI. As the primary outcome of the original study was based upon an evaluation of PCT, recruitment was limited to those children who were undergoing blood tests. As such, the proportion of children with an SBI diagnosis (24%) is higher than that of the population of all febrile children attending the ED, estimated (in children under 5) to be 7%[6]. This has been a common limitation of diagnostic accuracy studies of SBI in children, particularly those in which the focus has been on biomarker evaluations[185]. A recent external validation of published clinical prediction rules in nearly 10000 presentations of febrile children to primary care reported disappointing performance characteristics for most of the rules. In particular the rules demonstrated a limited ability to rule out serious illness[361]. There were particular challenges associated with undertaking the study, including the reliable collection of multiple clinical variables, and the appropriate attribution of outcomes. In this setting, where the determination of an outcome diagnosis was impractical, a proxy of 'referral to ED' was applied. This should be considered an important and appropriate proxy of serious outcome in febrile children, but it is unsurprising that prediction models derived to identify serious infection fail to explain additional variation in primary care referral practices. There remains a need to undertake high quality diagnostic accuracy studies in lower risk populations in both ED and in primary care. Such studies ought to identify the most parsimonious models, and identify ways – such as the use of electronic devices – to mitigate problems of missing data by automatically documenting clinically relevant variables.

Acknowledging the particular characteristics of our sample, the external validation of the Nijman *et al* prediction model provides support for its generalisability. Discrimination and calibration of the models for the diagnosis of pneumonia and other SBIs was good, and at risk thresholds of 20% the PLRs (6.5 and 5.1 for the diagnosis of pneumonia and other SBI respectively) supported a rule in decision, and at 5%, the NLRs (0.16 and 0.13 respectively) supported a rule out decision. This is the first such validation of the model that we are aware of. The Lab score has been externally validated now on two occasions[253,364]. The most recent validation reported a *c* statistic of 0.79 for the diagnosis of SBI and at a threshold score of 3 (as identified in the original study) sensitivity, specificity, PLR and NLR were 60%, 86%, 4.32 and 0.46 respectively. That is, a score of ≥3 provided some support to rule in SBI. Interestingly, as part of the external validation, the authors investigated the additional value of PCT or urinalysis over and above CRP (instead of the three together as proposed in the Lab score), and concluded that in children with either very low or very high CRP, the post-test probability was not substantially altered by the addition of these tests. In children with intermediate CRP values (between 20 and 100), the addition of a PCT result had the potential to significantly alter the likelihood of SBI[364]. The Brent score performed poorly in our sample and we are not aware of another external validation of this.

While the external validation provides reassurance of the reliability of the clinical predictions, the important consideration is the potential impact of these predictions upon clinical decision-making. This kind of impact analysis is the final stage of the process of translating diagnostic accuracy research into clinical practice, and is rarely achieved, particularly in children[234,363]. It is a branch of the emerging field of implementation science which seeks to understand how such evidence-based tools are adopted and sustained. Disappointingly, a decision model based upon the Nijman prediction model was recently evaluated in a randomised controlled trial of 439 febrile children (1 month to 16 years) in ED. While the model discrimination remained good, and compliance with the decisions was high, no significant improvement in outcomes (in terms of correct diagnoses, and also improvements in length of stay or hospital admission) were observed[371]. A small

trial of the Lab score similarly failed to demonstrate an impact on antibiotic prescribing in young children (less than 3 years)[370].

It is important to understand why these interventions, supported by robust evidence to demonstrate the reliability of their predictions, failed to impact clinical care. In the case of the Lab score, the authors explain the lack of impact as a consequence of a lack of adherence, and suggest that had adherence been higher, an estimated 26% reduction in antibiotic prescribing might have been observed[370]. In the case of the Nijman model, adherence to the protocol was good. The authors speculate that the lack of demonstrable impact from the decision rule intervention may be explained by a high level of diagnostic discrimination in the control group as a consequence of the setting in a specialist paediatric ED[371].

In both cases, the introduction of the diagnostic intervention relies upon more than simply the evidence demonstrating diagnostic accuracy. Translating this evidence from diagnostic accuracy studies into an effective clinical tool requires an understanding of the barriers preventing implementation. Such barriers may be logistical, behavioural, or economic and should be evaluated as part of studies which attempt to quantify impact. Understanding clinician behaviour regarding interventions to rationalise imaging in the ED[369], as well as to rationalise antibiotic prescribing has been important in achieving significant clinical improvements[376,377].

7.3. Clinical implications

Bacteraemia is a clinically important SBI associated with substantial morbidity and mortality. It occurred in 1.4 per 1000 non-trauma attendances to the ED in our dataset, and has been estimated to occur at a rate of 4 per 1000 in febrile children in this setting. It remains difficult to predict its presence or absence, still less identify a causative organism and its associated antimicrobial susceptibilities. Understanding the changing aetiology of children's bacteraemia in the ED has important implications for the establishment of appropriate empirical antibiotic regimens.

The increasing proportion of infections which are HCA means that empirical antibiotic regimens will need to take account of a broader range of potential pathogens, and appropriately cover possible resistant organisms. Identifying those children most likely to require broad antimicrobial cover will be necessary in order to make rational use of antibiotics. Evidence-based criteria for the definition of HCA bacteraemia would allow the targeting of those children most at risk.

The use of adjunctive diagnostic tests with the potential to expedite pathogen identification would support this objective of rational antibiotic use. Alongside a designated antimicrobial stewardship intervention, the use of MALDI-TOF MS (which applies mass spectrometry to positive culture isolates, Section 4.2.1) has been associated with a shortened time to effective antibiotics in adults with bacteraemia[303]. Our data do not support a role for SepsiTest at this time. SepsiTest is able to diagnose bacteraemia in small volumes of whole blood taken from febrile children in the ED. Its value in addition to blood culture is moderate, however, and we have not evaluated its ability to report positive results more quickly, and thereby influence clinical decision relating to antimicrobial use. It is likely that tests such as SepsiTest should be used in a targeted fashion in children considered most at risk of bacteraemia, and in whom empirical antibiotics have already been started. The ongoing cost-effectiveness review of SepsiTest alongside Septifast and Iridica (see Section 7.1.2) may be informative in this regard.

In the UK, the current approach to the febrile child presenting to the ED is supported by the clinical guideline "Feverish illness in children", published by NICE. This consensus guideline has been demonstrated to have limited capacity to discriminate between self-limiting and serious infections[352], and improved methods of risk assessment should be introduced as a priority.

Our data support a growing body of research to suggest that clinical prediction models can improve the prediction of risk of SBI in febrile children of all ages presenting to the ED. Combining predictive clinical signs with biomarkers such as CRP can accurately predict the presence of SBI. The addition of other biomarkers such as PCT and Resistin may further improve risk classification, but our evidence to support this is limited.

7.4. Research implications

The absence of a validated, evidence-based definition of HCA bacteraemia in children should be addressed through population-based studies which combine clinical and microbiological data. Such a definition would support clinicians to identify those children in the ED most likely to need broad empirical antibiotics.

Further evaluations of adjunctive tests to identify bacteraemia, such as SepsiTest, should be undertaken in well-defined populations of children. The potential impact of the introduction of such diagnostics is both in increasing the sensitivity of diagnosis beyond that of blood culture, and in identifying a clinically relevant reduction in time to pathogen identification which significantly impacts clinical care. In order to address these questions, studies should be undertaken in real-time, and should include a cost-effectiveness evaluation. Identifying suitable methods to define an appropriate reference standard (either by using a consensus diagnosis[378], or statistical methods to 'correct' the imperfect reference standard[64]) would allow a better understanding of the true value of these tests.

The risk classification improvement achieved by updating the model of Nijman *et al* requires validation in an external population. Though relatively modest, the

addition of the biomarkers PCT and Resistin to the Nijman model may be considered worthwhile if demonstrated to improve outcomes by improving time to antibiotics in those with SBI, and by reducing unnecessary hospital admission and antimicrobial use in those without. Recognising the challenges of determining outcome diagnoses in studies of SBI in children, appropriate outcomes for diagnostic accuracy studies should include practical, clinical end-points such as the need for hospital admission or antimicrobial use.

There is a significant need to evaluate the clinical impact of demonstrably accurate, validated risk models when incorporated into decision-making. Intervention studies of decision tools in this setting have so far been disappointing[371,374]. Evaluation of this clinical impact requires an understanding of the behaviour of clinicians, parents and children in using and interpreting such a proposed tool. Important considerations would include the determination of appropriate risk thresholds around which to base decisions, and what these decisions should be – admit, treat, observe, investigate, discharge? Recognising the residual risk inherent in the diagnostic process, another important area of research includes the most appropriate approach to safety netting – providing adequate support for children and families who are being discharged from the ED[48].

The role of biomarkers in published risk stratification models for the diagnosis of SBI in children is relatively modest. The model published by Nijman incorporated the use of CRP, while the Lab score combines CRP and PCT, alongside a urinalysis test. While CRP and PCT assays are both available as near-patient tests, in order to realise the potential of these biomarkers in the context of multivariable risk prediction, the development of multiplex bedside assays should be pursued. There is currently no near-patient test for Resistin.

The potential impact of improved risk prediction upon clinical decision-making may be substantial, though such an outcome has yet to be demonstrated. The increasing number of short stay, uncomplicated hospital admission in children with self-limiting acute infections illustrates the difficulty faced by clinicians in the ED. Disappointing recent evaluations of established consensus guidelines, such as the

NICE guideline, should prompt a move towards evidence-based rules to support decision-making in the ED. In light of the recent evidence from RCTs of the Lab score and Nijman model validated here, future studies of diagnostic interventions of this type require a multidisciplinary approach in order to understand the most effective methods of implementation. Only in the context of such implementation studies will it be possible to evaluate the impact of these diagnostic approaches.

7.5. Conclusions

This thesis details the persistent challenges relating to the diagnosis of SBI in the children's ED. We have described the evolving nature of paediatric bacteraemia, and the implications for both prompt recognition and effective empirical antimicrobial therapy. Our evaluation of the SepsiTest broad-range molecular diagnostic provides evidence of its potential use in whole blood samples from children in the ED, but highlights too its limitations in this setting. We have derived risk prediction models incorporating recognised clinical variables alongside the biomarkers CRP, PCT and Resistin, and externally validated previously published risk prediction models of SBI in the children's ED, estimating the additional value of the biomarkers PCT and Resistin to a model incorporating CRP alone. This work should provide further impetus for the evaluation of the impact of decision rules based upon such risk models upon important clinical outcomes such as admission to hospital, and rational antimicrobial use.

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Appendix 1: Information leaflet detailing the SPICED study provided to eligible older children

Do I have to take part?

No.

Can I change my mind?

Yes. If you change your mind, just tell your parent/guardian or the doctor or nurse.

Who is doing this research?

Doctors at your hospital and scientists at the University of Liverpool.

Who do I ask about this?

Your parents will have been given lots of information. If they are not sure, the doctor or nurse who gave you this information leaflet can tell you more.

UNIVERSITY OF LIVERPOOL

Alder Hey Children's NHS
NHS Foundation Trust

SPICED Study

Salivary **P**rocalcitonin for the Detection of Bacterial **I**nfection in **C**hildren admitted to the **E**mergency **D**epartment

Oldest Participants Information Leaflet (13 -16 years)

How do the doctors know if the germs are making you sick?

By looking at samples of blood and other fluids under a microscope or sending these samples to a laboratory for more tests, doctors can sometimes tell if you have a bacterial infection. This is not always possible and the tests we currently use may sometimes fail to pick up people with a serious infection.

What is the study about?

In this study we will be looking at all children and young people who come to A+E with a fever. We are a looking at a test to see if it can tell us if you have bacterial germs in your body. We will measure this test in your saliva (spit) as well as in your blood.

Why is the study being done?

If we can show that this new test is reliable at predicting bacterial infection in saliva as well as in blood then it can be used to help decide whether people need antibiotics or not, without the need for a blood test.

What will happen?

We will collect the saliva by putting a swab (cotton wool roll) in your mouth for a few minutes and then taking it out. It does not hurt at all. There will be no extra needle pricks as part of the study. We will do the tests on the blood sample that is already being taken from your arm or hand. The doctors or nurses will ask if you wish to have local anaesthetic cream on before they take the sample.

What will happen to the blood sample?

Your samples for will be tested in accredited laboratories at Alder Hey and at the Health Protection Agency. They will be kept safe in locked freezers and will only be identified by a code, for increased security. Once this study has been completed the sample will made anonymous, so it cannot be traced back to you.

Are there any risks or benefits to taking part?

There are no risks for you. We are hoping the information collected will help children/young people in the future.

Appendix 2: SPICED study form for recording parental consent

Alder Hey Children's NHS Foundation Trust
Liverpool
L12 2AP
Telephone: 0151 228 4811

Attach patient ID sticker here

Study Number:
Participation Identification Number for this study:

CONSENT FORM FOR RESEARCH
For parent/person with parental responsibility

Title of project: Salivary Procalcitonin In Children attending the Emergency Department (SPICED)

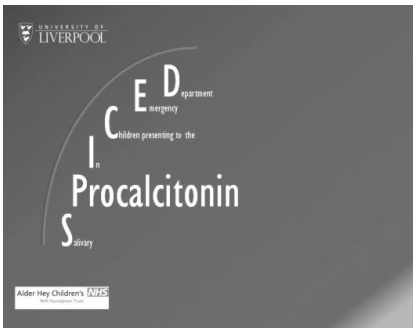
Name of researcher: **Job Title:**

	Please initial box
1. I confirm that I have read and understood the information sheet dated 10/08/10 (version 2.0) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily	
2. I understand that my child's participation is voluntary and that I am free to withdraw my child at any time, without giving any reason. If I do withdraw, his/her medical care and legal rights will not be affected in any way.	
3. I understand that relevant sections of my child's medical notes and data collected during the study may be looked at by individuals from regulatory authorities or from the NHS Trust, where it is relevant to my child taking part in this research. I give permission for these individuals to have access to my child's records	
4. I agree for my child to take part in the above study.	
5. I agree to my child's samples being stored for future studies.	

Name of patient _____

----------	----------	----------
Name of parent and Guardian	Date	Signature
Name of person taking consent (if different from researcher)	---------- Date	---------- Signature
Researcher	---------- Date	---------- Signature

Patient ID:



PROCEDURE CHECKLIST (Tick if done)

Signed consent [ ]
(white copy to parents, yellow with CRF, pink in notes)

Salivary PCT [ ]

Blood	FBC	[ ]
	Glucose	[ ]
	Blood culture	[ ]
	Blood PCR (0.5ml EDTA/red)	[ ]
	Blood PCT/NGAL (1ml U+E sample)	[ ]
CXR		[ ]
Urine	Urinalysis	[ ]
	Microscopy, Culture	[ ]

Date of admission ____/____/____

Date of discharge: ____/____/____

Discharge diagnosis: _____

Recruitment:	Nurse	Doctor

Name: _____ DOB: _____

Unit No: _____ Sex: M / F

Postcode _____

Date of admission ____/____/____ Time of booking (24 hour clock) _____

Route of Admission Direct to A+E / GP / other hospital

Sought medical care in previous 24 hrs Y N
If yes, from GP/walk-in/ NHS direct/A+E

Sought medical care in previous 48 hrs Y N
If yes, from GP/walk-in/ NHS direct/A+E

Any antibiotic given prior to admission to A+E Y N

Antibiotic	Dose	Frequency	Start date dd/mm/yy

<u>Past Medical History:</u>

Preceding Illness Y / N
ICD 10 code: _____

Chronic Illness Y / N
ICD 10 code: Primary _____
Secondary _____

Feeding: Breast / Formula / Breast+Formula / Weaned / Weaned+Breast

Parental Smoking Y / N / NR

Clinical History:

	Y/N	Duration (hrs)
Fever		
Vomiting		
Diarrhoea		
Drowsiness		
Headache		
Rash		
Myalgia		
Irritability/ Inconsolable crying		
Dysuria		
Abdo pain		
Decreased intake		
Dry nappies/ reduced UO		

	Y/N	Duration (hrs)
Cough		
Difficulty breathing		
Wheeze		
Chest pain		
Sore throat		
Ear ache		
Seizures		
Neck Stiffness		
Parental concern		

Other Y/ N State other_____

<u>Immunisation</u>

2 months	DTaP/IPV / Hib	Y/N	PCV	Y/N
3 months	DTaP/IPV / Hib	Y/N	MenC	Y/N
4 months	DTaP/IPV / Hib	Y/N	MenC	Y/N
			PCV	Y/N
12 months	Hib	Y/N	MenC	Y/N
13 months	MMR	Y/N	PCV	Y/N
3-4 yrs	DTaP/IPV	Y/N	MMR	Y/N
Girls 12-13 years	HPV	Y/N		
13-18 years	dT/IPV	Y/N		

Clinical Examination

General appearance	Very unwell/moderately unwell/mildly unwell
Bulging fontanelle	Yes/no/closed
Rash	Yes/no If yes petechial/purpuric/maculopapular/other
If other, describe	
Abn ENT signs	Y/N
Sunken eyes	Y/N
Dry mucous membranes	Y/N
Pale skin	Y/N

Dehydration Y N If yes, mild Y N, moderate Y N, severe Y N
Oedema Y N Jaundice Y N

Respiratory System

Cyanosis	Y	N	If no to the above:		
↑ Work of breathing	Y	N	Normal air entry	Y	N
Stridor	Y	N	Chest clear	Y	N
Decreased breath sounds	Y	N			
Crackles	Y	N			
Wheeze	Y	N			

Signs RML / RLL / RUL / LLL / LUL
Finger clubbing Y N

Cardiovascular System

Cyanosis Y N
HS normal Y N
If no, Murmur / third sound / other

Abdomen

Oral thrush	Y	N	Mouth sores	Y	N
Liver enlarged	Y	N	If yes,	cm below RCM	
Spleen enlarged	Y	N	If yes,	cm below RCM	

Lymphadenopathy Y / N
If yes, cervical / submandibular / axillary / inguinal / epitrochlear

Neurological
Mental state: A / V / P/ U

Pupillary reflex: PERL/abnormal
If abnormal, describe:
Neck stiffness: Y / N
Kernig's sign: Y / N

Fundoscopy: Done / Not done
Fundoscopy: Normal
Blurred disc margins
Papilloedema
Haemorrhage
Not visualised

Overall clinical impression:

Fever without source Y / N

Other: _____

Time Study:

Hr	Temp	SBP	DBP	MAP	HR	CR	AVPU	RR	Sats
0									
1									
2									
3									
4									
5									
6									

Time antibiotic given (24 hr clock) _ _:_ _

Fluids given in first 6 hours (circle all that apply)

Normal saline / albumin / gelofusin / blood /platelets / fresh frozen plasma

Please complete fluids form

OUTCOME

Duration of hospital stay (days):

PICU admission Y N
If yes, date of PICU admission _____ date of PICU discharge_____

HDU admission Y N
If yes, date of HDU admission _____ date of HDU discharge_____

Dead/Alive Date of death: _____

Final diagnosis
ICD 10 code 1. Primary _____
2. Secondary _____

Antibiotic form Weight:

Antibiotic	Route	Dose	Frequency	Start date dd/mm/yy	Stop date dd/mm/yy

Route: IV=intravenous, O=oral, Neb=Nebulised, IM=intramuscular, T=topical, IT=intrathecal, SDD= selective decontamination of digestive tract

Intravenous Fluid Form

Fluid	Bolus (B) or Infusion (I)	Infusion rate (ml/hr)	Start time 24 hr clock	Stop time 24 hr clock

Etiology of Childhood Bacteremia and Timely Antibiotics Administration in the Emergency Department

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abstract

BACKGROUND: Bacteremia is now an uncommon presentation to the children's emergency department (ED) but is associated with significant morbidity and mortality. Its evolving etiology may affect the ability of clinicians to initiate timely, appropriate antimicrobial therapy.

METHODS: A retrospective time series analysis of bacteremia was conducted in the Alder Hey Children's Hospital ED between 2001 and 2011. Data on significant comorbidities, time to empirical therapy, and antibiotic susceptibility were recorded.

RESULTS: A total of 575 clinical episodes were identified, and *Streptococcus pneumoniae* ($n = 109$), *Neisseria meningitidis* ($n = 96$), and *Staphylococcus aureus* ($n = 89$) were commonly isolated. The rate of bacteremia was 1.42 per 1000 ED attendances (95% confidence interval: 1.31–1.53). There was an annual reduction of 10.6% (6.6%–14.5%) in vaccine-preventable infections, and an annual increase of 6.7% (1.2%–12.5%) in Gram-negative infections. The pneumococcal conjugate vaccine was associated with a 49% (32%–74%) reduction in pneumococcal bacteremia. The rate of health care–associated bacteremia increased from 0.17 to 0.43 per 1000 ED attendances ($P = .002$). Susceptibility to empirical antibiotics was reduced (96.3%–82.6%; $P < .001$). Health care–associated bacteremia was associated with an increased length of stay of 3.9 days (95% confidence interval: 2.3–5.8). Median time to antibiotics was 184 minutes (interquartile range: 63–331) and 57 (interquartile range: 27–97) minutes longer in Gram-negative bacteremia than in vaccine-preventable bacteremia.

CONCLUSIONS: Changes in the etiology of pediatric bacteremia have implications for prompt, appropriate empirical treatment. Increasingly, pediatric bacteremia in the ED is health care associated, which increases length of inpatient stay. Prompt, effective antimicrobial administration requires new tools to improve recognition, in addition to continued etiological surveillance.

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Dr Irwin designed the study, collected data, performed the analysis, and drafted the original manuscript; Drs Carrol and Drew designed the study and reviewed and revised the manuscript; Dr Diggle designed the study, supervised the data analysis, and reviewed and revised the manuscript; Dr McNamara reviewed and revised the manuscript; and Dr Marshall, Dr Nguyen, Dr Hoyle, Dr Macfarlane, Dr Wong, Dr Mekonnen, Dr Hicks, Dr Steele, Ms Gerrard, and Ms Hardiman collected and cleaned the data and reviewed the manuscript; and all authors approved the final manuscript as submitted.

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WHAT'S KNOWN ON THIS SUBJECT: Childhood bacteremia caused by vaccine-preventable organisms has substantially declined over the last decade. Recognition of bacteremia in children is difficult, and delayed administration of antibiotics is associated with poor outcomes. Adults with health care–associated Gram-negative bacteremia experience delays in receiving appropriate antibiotics.

WHAT THIS STUDY ADDS: Bacteremia in children presenting to the emergency department is increasingly health care associated and resistant to empirical antibiotics. These infections are associated with increased length of stay. Rates of Gram-negative bacteremia have increased, and children with Gram-negative bacteremia experience delayed antibiotic administration.

Acute infections are a common reason for presentation to the children's emergency department (ED).[1] In young children presenting with an acute febrile illness, serious bacterial infection occurs in ~7%. Bacteremia occurs in 1 in 250 febrile children aged <5 years and may be difficult to recognize.[2]

Bacteremia is associated with significant mortality and morbidity in children. Immediately before the introduction of the pneumococcal vaccine in the United Kingdom, 20% of childhood mortality was infection related, with "septicemia" the most commonly documented cause of death.[3] The etiology of pediatric bacteremia in the United Kingdom has evolved substantially as the immunization schedule has expanded. The incidence of vaccine-preventable infections has declined,[4] while that of Gram-negative infections has increased.[5]

Timely, effective antimicrobial therapy is fundamental to the management of serious infections in children. Delayed recognition of meningococcal disease,[6] and suboptimal resuscitation of septic shock, contribute to mortality in children.[7,8] The Surviving Sepsis Campaign recommends administration of antibiotics within 1 hour of recognition of severe sepsis.[9]

Historically, serious infections have been categorized according to the timing of their identification into "community-acquired" and "hospital-acquired" infection. These categories were used to inform the likely etiology and to guide empirical treatment. There is an increasing awareness of patients who, although they acquire serious infections in the community, share the characteristics of patients with "hospital-acquired" infections. Such occurrences have been termed health care–associated (HCA) infections.[10] In adults, HCA bacteremia is associated with delayed administration of appropriate antibiotics.[11] To date, no validated definition of HCA bacteremia is in use in children.[12]

The objective of the present study was to describe the etiology of bacteremia by using data from children presenting to the Alder Hey Children's Hospital ED between 2001 and 2011. Our goal was to explore the impact of temporal changes on outcomes, including the timeliness and appropriateness of empirical antibiotic therapy.

METHODS

A retrospective time series analysis was conducted of all clinically significant episodes of bacteremia presenting via the Alder Hey Children's Hospital ED. This children's ED is the busiest in the United Kingdom, with ~60 000 attendances per year. All positive results of blood culture isolates from 2001 to 2011 (inclusive) were identified. Positive culture results isolated >48 hours after presentation, or associated with a hospital admission in the previous month, were considered hospital-acquired and excluded. Isolates commonly considered commensals were included if they were cultured on >1 occasion within 48 hours.[13]

Clinical data relating to each episode were extracted from clinical notes and the hospital electronic database. Recorded data included demographic characteristics and significant comorbidities, including the presence or absence of a central venous line (CVL). Commonly used markers of infection (white blood cell count, neutrophil count, platelets, and C-reactive protein) were extracted electronically.

To examine trends and to compare our data with published evidence, we grouped episodes of *Streptococcus pneumoniae, Neisseria meningitidis,* and *Haemophilus influenzae* as vaccine-preventable infections; Gram-negative infections were similarly grouped. For the remaining Gram-positive infections, we differentiated between typically pathogenic organisms in healthy children such as *Staphylococcus aureus* (typical Gram-positive) and those considered to be associated with the health care setting, such as coagulase-negative *Staphylococcus* (ie, other Gram-positive).

Definitions

Community-acquired bacteremia was defined as the identification of a significant pathogen in a blood culture taken within 48 hours of presentation to the ED (in the absence of admission to the hospital in the previous month).

HCA bacteremia was defined as the identification of a significant pathogen in a blood culture taken within 48 hours of presentation to the ED in the following children: those with an indwelling device (eg, CVL, ventriculoperitoneal shunt, prosthetic material); those with primary or acquired immunodeficiency; those requiring regular hospital-based intervention (eg, hemodialysis, intravenous therapies); and preterm infants <12 months from discharge from the neonatal unit.

Empirical therapy was defined as therapy instituted before microbiologic evidence of infection, limited to 24 hours from presentation to the ED. Therapy instituted beyond this period, or where there was documentation of microbiological advice, was termed "directed."

Susceptibility to empirical therapy was defined as susceptibility to the empirical therapy initiated by the clinical team. Susceptibility was determined by breakpoints in use at the time of isolation, according to methods of the British Society for Antimicrobial Chemotherapy.[14]

Statistical Methods

Statistical analysis was undertaken in R version 3.0.1.[15] A detailed explanation of the statistical methods is described in the Supplemental Appendix.

Continuous data were described according to medians and interquartile ranges, and comparison between groups was performed by using the Kruskal-Wallis test. Categorical variables were expressed as percentages with 95% confidence intervals (95% CIs). Groups were compared by using the χ^2 test.

Time Series Analysis

Poisson regression was used to model weekly counts of bacteremia with weekly ED attendance as an offset. Logistic regression was used to model the likelihood over time (ie, the fitted likelihood) that each clinical episode occurred in a child with an indwelling CVL or that the isolate was susceptible to empirical antibiotic therapy.

Inspection of these models suggested an initial increase in the likelihood of a clinical episode occurring in a child with an indwelling CVL, with a subsequent reduction from 2008. In parallel, there was an initial reduction in the likelihood of an isolate being susceptible to empirical antibiotics, with an increase after 2008. Late in 2007, the hospital increased investment in specialist intravenous nurses, with responsibility for training in the management of CVLs. We considered this intervention to be a plausible explanation for the observed variation, and we implemented a piecewise fit of time with the breakpoint specified according to the time of the clinical intervention. Inclusion of the piecewise variable improved the fit of the model to the data.

Multivariable Analysis of Clinical Outcomes

Length of stay (LOS) and time to empirical antibiotic administration were assessed by using multivariable linear regression. Both factors were log-transformed to meet the assumption of linearity between outcome and explanatory variables. In the model assessing time to antibiotic administration, a piecewise fit for platelets was undertaken and found to improve the fit of the model. A maximal model of all variables with a P value <.1 was fit before model simplification was undertaken in a backward stepwise process.

The use of anonymized data was approved by the research department of the Alder Hey Children's Hospital.

RESULTS

Between 2001 and 2011, a total of 692 clinically significant blood cultures were identified in children sampled within 48 hours of presentation to the ED. These cultures represented 575 episodes of bacteremia in 525 children.

Clinical Characteristics of Children

The characteristics of the 525 children presenting with bacteremia are summarized in Table 1. Significant comorbidities were present in 151 children (29%). The most common comorbidities were gastrointestinal (Supplemental Table 5), and many of these children (22 of 25) had indwelling CVLs for the purpose of parenteral nutrition. Children with Gram-negative isolates were younger and more likely to have significant comorbidities or an indwelling CVL than those with vaccine-preventable or typical Gram-positive isolates. Mortality in children with other Gram-positive infections was higher (6 of 37) than in other groups (odds ratio: 5.15 [95% CI: 1.60–15.8] vs vaccine-preventable isolates).

Children with HCA bacteremia were older than those with community-acquired bacteremia. LOS in children with HCA bacteremia was prolonged by 3.9 days versus community-acquired bacteremia (95% CI: 2.3–5.8). Mortality and admission to the PICU did not differ between these groups (Table 2).

Etiology of Bacteremia in the Children's ED

Isolated organisms varied according to age (Fig 1). In early infancy, the most common organisms were *Escherichia coli* and Group B streptococcus. There were 47 presentations of neonatal sepsis, including 1 of early-onset disease (Group B streptococcus). *S pneumoniae* was most common overall. *S pneumoniae* and *N meningitidis* occurred more

TABLE 1 Clinical Characteristics of 525 Children Presenting to the ED With Bacteremia According to Type of Organism

Variable	Median	IQR	Vaccine-Preventable (n = 221)	Typical Gram-Positive (n = 149)	Gram-Negative (n = 118)	Other Gram-Positive (n = 37)	P
Age, y	1.32	0.42–4.27	1.80 (0.83–3.88)	1.58 (0.13–6.82)	0.82 (0.20–3.08)	1.64 (0.69–11.45)	<.001[a]
LOS, d	6	3–10	6 (4–9)	7 (3–13)	6 (4–10)	7 (2–11)	.5[a]
	%	95% CI	*n* (%)	*n* (%)	*n* (%)	*n* (%)	
CM	28.8	25.1–32.8	36 (16.3)	42 (28.2)	51 (43.2)	22 (59.5)	<.001[b]
CVL	9.0	6.80–11.7	3 (1.40)	6 (4.02)	26 (22.0)	12 (32.4)	.001[c]
PICU	18.9	15.8–22.5	56 (25.3)	23 (15.4)	13 (11.0)	7 (18.9)	.007[b]
Mortality	4.57	3.09–6.71	8 (3.62)	6 (4.02)	4 (3.39)	6 (16.2)	.005[c]

CM, significant comorbidity; IQR, interquartile range.
[a] Kruskal-Wallis test.
[b] χ^2 test.
[c] Monte Carlo simulation.

TABLE 2 Clinical Characteristics of Community-Acquired Bacteremia and HCA Bacteremia

Characteristic	Community-Acquired (n = 444)	HCA (n = 81)	P
Median age, y (IQR)	1.43 (0.35–3.88)	2.32 (0.96–6.48)	.001[a]
Median LOS, d (IQR)	6 (3–9)	9 (4–18)	<.001[a]
PICU, % (95% CI)	19.0 (15.6–22.9)	18.5 (11.6–28.3)	.15[b]
Mortality, % (95% CI)	4.1 (2.6–6.3)	7.4 (3.4–15.2)	.25[c]
Group			<.001[b]
Vaccine-preventable	207	14	
Typical Gram-positive	133	16	
Gram-negative	82	36	
Other Gram-positive	22	15	
Median time to antibiotics, min (IQR)	181 (59–321)	218 (91–353)	.15[a]

IQR, interquartile range.
[a] Kruskal-Wallis test.
[b] χ^2 test.
[c] Monte Carlo simulation.

commonly between the ages of 1 and 5 years, whereas *S aureus* was the most common organism in children aged >5 years. Most meningococcal isolates (87 of 96) were Group B; the remainder were Groups C (n = 5), W135 (n = 3), and Y (n = 1). Two episodes of methicillin-resistant *S aureus* bacteremia occurred over the 11-year period; 1 episode was associated with ventriculitis in a child with a ventriculoperitoneal shunt, and 1 episode was in a previously well child. There were 17 polymicrobial infections in 13 children; 12 had significant comorbidities, and 8 had CVLs in situ. Isolated organisms are summarized in Table 3.

Time Series Analysis

The rate of clinically significant bacteremia in children presenting to the ED was 1.42 per 1000 attendances (95% CI: 1.31–1.53). The cumulative frequency plot reveals a declining rate of vaccine-preventable isolates, including *S pneumoniae*, and an increasing rate of Gram-negative isolates (Supplemental Fig 4).

Poisson regression was used to model the observed rate of bacteremia (Fig 2). For the overall rate of bacteremia, neither the trend over time (P = .18) nor the seasonal effect (P = .17) was statistically significant. Bacteremia caused by vaccine-preventable isolates including *S pneumoniae* was highly seasonal (P < .001). From 2001 to 2011, its rate declined from 1.32 to 0.37 per 1000 ED attendances at an annual rate of reduction of 10.6% (95% CI: 6.6–14.5). The pneumococcal conjugate vaccine (PCV) was introduced into the UK immunization schedule in September 2006. When incorporated into the regression model, PCV was associated with a 49% reduction in pneumococcal bacteremia (95% CI: 32–74) from 0.50 to 0.25 per 1000 attendances. By contrast, the rate of Gram-negative bacteremia increased from 0.24 to 0.53 per 1000 ED attendances (P = .007). The fitted seasonal effect in the Gram-negative model, although not statistically significant (P = .07), exhibited a peak in summer, in contrast to that of the vaccine-preventable model, which peaked in winter.

The rate of community-acquired bacteremia was reduced from 0.93 to 0.57 per 1000 ED attendances (P = .005) between 2001 and 2011, while the rate of HCA bacteremia increased from 0.17 to 0.43 per 1000 (P = .002). The proportion of clinical episodes occurring in children with an indwelling CVL increased from 3.2% in 2001 (95% CI: 1.47–6.84) to a peak of 26.5%, before declining to 21.8% in 2011 (95% CI: 12.9–34.3). In parallel, the likelihood that an isolate was susceptible to empirical therapy was reduced from 96.3% (95% CI: 92.1–98.2) to 82.6% (95% CI: 69.8–90.7) between 2001 and 2011; it reached a nadir of 74.4% in 2008. These trends are illustrated in Fig 3. LOS, likelihood of PICU admission, and mortality did not change over time.

Timeliness and Appropriateness of Empirical Antibiotics

The empirical antibiotic protocol was documented in 563 of 575 clinical

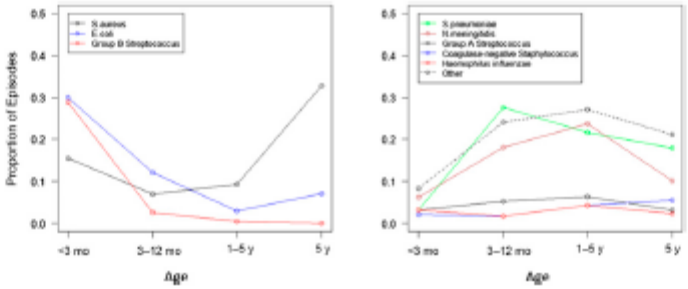

FIGURE 1
Proportion of bacteremia episodes of each isolate according to age group.

TABLE 3 Predominant Organisms According to Group

Organism	*N*	%
Vaccine preventable, *n* = 223		
S pneumoniae	109	19.0
N meningitidis	96	16.7
H influenzae (type B)	18	3.1
Typical Gram-positive, *n* = 149		
S aureus	89	15.4
Group B streptococcus	32	5.6
Group A streptococcus	28	5.9
Gram-negative, *n* = 152		
E coli	59	10.3
Klebsiella sp	26	4.5
Acinetobacter sp	11	1.9
Enterobacter sp	10	1.7
Pseudomonas sp	10	1.7
Salmonella sp	9	1.6
Moraxella catarrhalis	9	1.6
Other	18	3.2
Other Gram-positive, *n* = 51		
Coagulase-negative *staphylococcus*	21	3.5
Enterococcus sp	19	3.3
Nonpyogenic *streptococcus*	6	1.0
Other	5	0.9

episodes (Supplemental Table 6). For children with no indwelling CVL, 375 of 480 received monotherapy (most commonly, cefotaxime [289 of 393]). In combination treatments, cefotaxime was also invariably used, along with gentamicin, benzylpenicillin, or amoxicillin. In children with an indwelling CVL, combination therapy was commonly used (46 of 83), particularly with teicoplanin and gentamicin.

We were able to define empirical susceptibility in 527 of 575 cases. Vaccine-preventable isolates were almost universally susceptible to empirical therapy (217 of 219), whereas 22% (29 of 131) of Gram-negative isolates were resistant ($P <$ .001). Data on time to antibiotic administration were available for 78% (444 of 575) of all episodes. Median time to antibiotic administration was ~3 hours and varied according to the type of organism (Table 4). Children with vaccine-preventable infections received empirical antibiotics more quickly than children with other types of infection.

To explore this relationship, a multivariable model of time to antibiotics was developed (Supplemental Table 7). After adjustment for other explanatory variables, time to antibiotics was increased by 57 minutes for Gram-negative infections compared with vaccine-preventable infections. Older children received antibiotics later than younger children. Overall, time to antibiotics increased by ~3 minutes per year of the study (P = .006 for linear trend).

DISCUSSION

In describing the changing etiology of bacteremia in the busiest children's ED in the United Kingdom, we have shown a reduction in vaccine-preventable bacteremia, coincident with an increase in Gram-negative bacteremia. The rate of pneumococcal bacteremia has been halved since the introduction of the PCV. These trends affirm data from surveillance studies of invasive bacterial infections internationally.[4,5,16–18] We identified a seasonality to Gram-negative bacteremia with a summer peak; although not statistically significant, this finding is consistent with other published data.[19,20]

Our analysis illustrates the changing characteristics of children presenting to the ED with bacteremia. Increasingly, these are children with underlying comorbidities and indwelling CVLs. The rate of HCA bacteremia is increasing. The proportion of episodes involving children with CVLs increased to a peak in 2008 before declining toward the end of the study. This finding occurred in parallel with an initial reduction in antibiotic susceptibility, which also improved after 2008. This outcome likely reflects more children with CVLs receiving total parenteral nutrition in the community. Unpublished data from Alder Hey Children's Hospital (S. Melville, personal communication, 2013) reveals a threefold increase in CVL-associated infection rates with the use of total parenteral nutrition (from 3.3 to 10.4 per 1000 line-days). At the end of 2007, the hospital increased investment in specialist intravenous nurses, with responsibility for training in the management of CVLs. This intervention may explain the later reduction in children with CVL-associated infections presenting to the ED.

The changing etiology of childhood bacteremia in the ED was not associated with temporal changes in mortality or PICU admission. HCA bacteremia was associated with an increased LOS, irrespective of the responsible organism. Other investigators have related increased

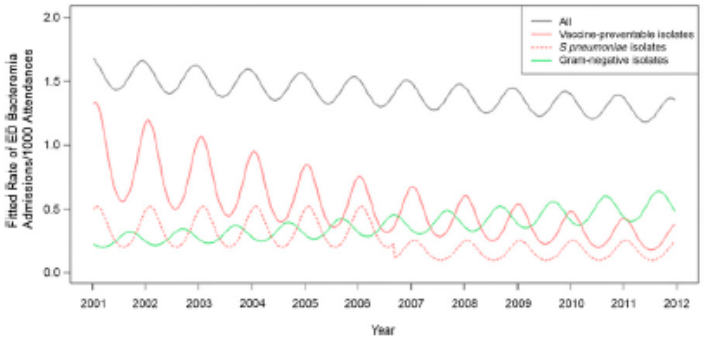

FIGURE 2
Fitted rate of pediatric bacteremia per 1000 attendances to the Alder Hey Children's Hospital ED between 2001 and 2011. The discontinuity in the fit for *S pneumoniae* is due to the introduction of the PCV in September 2006.

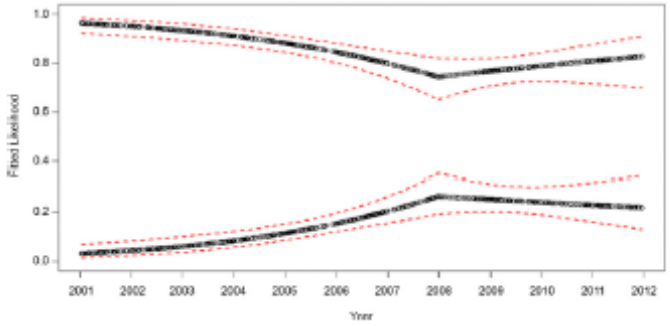

FIGURE 3

Fitted likelihood over time that an isolate was susceptible to empirical therapy (top) and that an episode occurred in a child with an indwelling CVL (bottom). Time was fit as a piecewise term to incorporate a clinical intervention. Inclusion of the piecewise term improved the model fit. Dotted red lines are 95% CIs.

LOS in adults to HCA bacteremia,[21] but we are unaware of pediatric data detailing the same. To date, this category of infection remains poorly defined in children.

Despite acceptance that time to antibiotic administration influences outcome in sepsis,[22] few studies have reported time to antibiotic administration in the pediatric ED.[23,24] We found that the changing nature of bacteremia in the pediatric ED has implications for both recognition and management. A median time to antibiotic administration of 3 hours is comparable with other data.[25] Empirical therapy took longer to initiate in children with Gram-negative infections than in children with vaccine-preventable infections, even with adjustment for other explanatory variables. Furthermore, multivariable modeling allowed us to estimate an increase in time to antibiotics of 38 minutes over the 11 years of the study, regardless of changes in etiology. These changes are likely multifactorial, but the implication is that additional resources in diagnostics and training will be required to minimize delays in the treatment of these most invasive infections.

Over 11 years, susceptibility to empirical antibiotic protocols declined. Adults with Gram-negative HCA bacteremia experience delays in appropriate antibiotic therapy,[11] and mortality is increased when initial empirical therapy is inadequate.[26] Death in children is uncommon even in serious infection, and we failed to draw an association between death or likelihood of PICU admission and inappropriate empirical antibiotics.

The present study had several limitations. It was a retrospective, single-center study of culture-positive bacteremia conducted in the pediatric ED. Blood culture yield is affected by the use of previous antibiotics (more so than polymerase chain reaction assays).[27] Collecting data on previous antibiotics was impractical in this retrospective analysis. Community antibiotic prescribing for children in the United Kingdom decreased substantially in the 1990s,[28,29] although some data suggest an increase through the course of the present study.[30] This finding would not account for the reduction in pneumococcal bacteremia, for which there was no temporal trend before the introduction of the PCV. An increase in prehospital antibiotic use may have affected blood culture yield over time, however, particularly if this use was well targeted toward children with bacteremia.

We have not accounted for changes to the population of the hospital catchment area but have instead incorporated ED attendance. Over time, there was no change in medical attendances to the ED.

Based on the published literature, we developed a pragmatic definition of HCA bacteremia.[12] To date, no such definition has been established in pediatric medicine. We identified a number of surrogate markers of frequent exposure to health care environments but were unable to collect robust data to quantify this occurrence in terms of how frequently or how recently exposure occurred in individual clinical episodes. Understanding this exposure better, thereby establishing a valid definition of HCA bacteremia, would help to guide empirical treatment in the ED. Because our definition was applied consistently across the 11 years of the data set, we believe our description of temporal trends is robust.

Data regarding time to antibiotics were missing in ~20% of clinical cases. We restricted our analysis to time to empirical therapy, although others have reported time to "appropriate" therapy.[11] We explored explanatory variables that may influence time to antibiotics and which were available in the clinical

TABLE 4 Time to Antibiotics According to Type of Organism

Organism	Median, min	IQR	*P*
All	184	62.5–330.5	
Vaccine-preventable	130	34.5–297	<.001
Typical Gram-positive	256	118–376	
Gram-negative	184	95.5–356	
Other Gram-positive	253.5	147–401	

IQR, interquartile range.

notes. Other studies have used validated measures of disease severity (eg, the Glasgow Meningococcal Septicaemia Prognostic Score) and demonstrated an association with time to antibiotic administration.[23] A comparable measure of illness severity may help to explain the variation reported, although no such tool is currently in use in all acutely unwell children in the ED.

We assumed that variation in time to antibiotics was related to clinical recognition. Time to antibiotics is also used as a measure of crowding in EDs. Some investigators have identified an association between markers of ED crowding and time to antibiotic administration in young infants,[31] although not consistently.[32] We did not collect data on ED crowding. There was no overall increase in numbers presenting to the Alder Hey Children's Hospital ED over the course of the study, nor was there an association between time of day, day of the week, or time of year (all factors that are associated with the volume of ED activity) and time to antibiotic administration.

CONCLUSIONS

We describe the evolving etiology of children with bacteremia presenting to the UK's busiest children's ED. Increasingly, these infections are considered HCA. They are more likely to be resistant to empirical therapy, more difficult to recognize, and are associated with a prolonged LOS. Prompt and effective antimicrobial treatment of bacteremia requires improved diagnostic tools in addition to continued etiological surveillance.

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