ABSTRACT

Introduction Early recognition and appropriate management of paediatric sepsis are known to improve outcomes. A previous system’s biology investigation of the systemic immune response in neonates to sepsis identified immune and metabolic markers that showed high accuracy for detecting bacterial infection. Further gene expression markers have also been reported previously in the paediatric age group for discriminating sepsis from control cases. More recently, specific gene signatures were identified to discriminate between COVID-19 and its associated inflammatory sequelae. Through the current prospective cohort study, we aim to evaluate immune and metabolic blood markers which discriminate between sepsis (including COVID-19) from other acute illnesses in critically unwell children and young persons, up to 18 years of age.

Methods and analysis We describe a prospective cohort study for comparing the immune and metabolic whole-blood markers in patients with sepsis, COVID-19 and other illnesses. Clinical phenotyping and blood culture test results will provide a reference standard to evaluate the performance of blood markers from the research sample analysis. Serial sampling of whole blood (50 μL each) will be collected from children admitted to intensive care and functional analysis will be performed during the hospital journey and in cases of suspected sepsis to investigate time-dependent changes in biomarkers. An integrated lipidomics and RNASeq transcriptomics analyses will be conducted to evaluate immune-metabolic networks that discriminate sepsis and COVID-19 from other acute illnesses. This study received approval for deferred consent.

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ Identification of host response molecular patterns associated with various infections (bacterial, viral and fungal).
⇒ Immunophenotyping, immunoassays, lipidomics and functional analysis will be performed during the hospital journey and in cases of suspected sepsis to investigate time-dependent changes in biomarkers of sepsis.
⇒ Potential selection bias towards younger cases.
⇒ Single site observational study.

INTRODUCTION

Sepsis is a global health problem affecting all age groups. A systematic review of published literature from 1979 to 2016 was carried out to estimate population-based sepsis incidence...
in neonates and children. Reports from 15 studies in 12
countries showed an aggregate estimate of 48 (95% CI
27 to 86) sepsis cases and 22 (95% CI 14 to 33) severe
sepsis cases in children per 100 000 person-years. The
population-level estimate for neonatal sepsis was 2202
(95% CI 1099 to 4360) per 100 000 livebirths, with
mortality between 11% and 19%. Extrapolating on a
global scale gave an estimated incidence of approximately
3 million cases of sepsis in neonates and 1.2 million cases
in children annually. Mortality ranged from 1% to 5% for
sepsis and 9% to 20% for severe sepsis.4

Definition of sepsis in children
At present, sepsis is defined in adults as life-threatening
organ dysfunction resulting from a dysregulated host
response to infection.2 This definition has removed the
terms ‘systemic inflammatory response syndrome’ (SIRS)
and ‘severe sepsis’ found in previous definitions and
highlights organ dysfunction for clinical management. In
children, the existing surviving sepsis consensus confer-
ence definition of sepsis based on the criteria for SIRS in
the presence of confirmed or suspected infection is still
used for identification of sepsis and clinical research.3
The recently updated guidance for the management of
sepsis in children (2020) recognises the clinical hetero-
genesis of this illness in children, accounting for life-
threatening organ dysfunction.4 A revised definition of sepsis by the paediatric sepsis definition task force is
awaited.5 Recently, we put forward a setpoint mechanism
for understanding sepsis. This suggests that dysregula-
tion of the host response to infection in sepsis is explain-
able by regulatory shifts in homeostasis setpoints. This
is due to negative and positive feedback changes in the
concerted behaviour of multiple (immune, metabolic and
neuronal) systems in response to infection. We hypothe-
sise that infants and children have age-dependent meta-
bolic demands and a developing immune setpoint that
increases the risk of sepsis.5 Sepsis can arise from any
type of infection—bacterial, viral, fungal or parasitic.4
Even though less common in children, COVID-19 can
be associated with severe illness. History of infection and
age-dependent changes in the immune system give rise
to subtle but important differences in response to SARS-
CoV-2 infection in children when compared with adults.
In the early life and younger years, the innate response is
a dominant feature of the immune response to infection.
In the case of SARS-CoV-2 infection, children are known
to mount a stronger and more vigorous interferon (IFN)
antiviral response than adults, curtailing viral replication
at an early stage of infection.7

Biomarkers in sepsis
Several host inflammatory biomarkers have been
suggested to improve the ability for early recognition of
sepsis. These include C reactive protein, procalcitonin,
interleukin 8, soluble CD14 (presenilin) and triggering
receptor expressed on myeloid cells that have all been
evaluated without conclusive results.8 It is important to
recognise that sepsis is a complex pathophysiological
process comprising an immune response which interacts
with the body’s metabolic and physiologic functions. A
profile of multiple markers capturing the complexity of
the pathophysiological response would be better than an
individual marker to detect and study the disease process.9
In neonatal sepsis, it has been shown that the inclusion of
metabolic markers as well as adaptive immune responses
with innate immune signal changes, increased the accu-
rocity for early recognition of sepsis.10 These complex
molecular changes during sepsis correlate with altered
gene expression11 and in some infectious illnesses, there
was a unique signature alteration in gene expression
with a demonstrable value in understanding pathoge-
nicity.12–14 Gene expression markers have been evaluated
to identify bacterial infections in febrile children15 and
differences in transcriptomic profiles in blood have been
used to discriminate clinical phenotypes and subgroups
of sepsis.16 17

COVID-19 in children and young people admitted to paediatric
intensive care
Infection with SARS-CoV-2 is mild or asymptomatic in
the majority of children.18 19 The proportion of children
presenting to the hospital who have severe COVID-19
and require admission to intensive care has been vari-
able ranging from 1.75% in China20 to a higher rate
of 9.7%–28% in the western world.21–23 The clinical
risk factors for severe COVID-19 in children have been
reported to be age over 10 years, black and Asian ethnic
groups, comorbidities and obesity.24 25 The difference in
the severity of COVID-19 in children, when compared
with adults and the elderly, has been studied exten-
sively. Various factors to explain this difference have
been reported and include vasculopathy, the density of
ACE 2 receptors, age-based differences in coagulation
profiles and immune responses,25 and heightened anti-
viral IFN response.7 Paediatric inflammatory multisystem
syndrome temporally related to SARS-CoV-2 (PIMS-TS)
also called multisystem inflammatory syndrome in
children is a hyperinflammatory syndrome seen in some
children following contact with SARS-CoV-2. Two phenotypes
have been described—high fevers with severe gastrointes-
tinal symptoms; and shock with myocardial dysfunction
and Kawasaki disease-like clinical features. Research to
evaluate the pathophysiology of PIMS-TS reported an
abnormal immune response mediated by superantigen
activity of the SARS-CoV-2 S glycoprotein.26 It has also
been associated with immune markers related to endo-
thelial dysfunction in children.27 Through this study, we
hope to compare immune responses in children with
COVID-19, PIMS-TS and sepsis.

Study methods for molecular and cellular analysis
The studies so far have been restricted to using tran-
scriptomic analysis for an observational case-control
methodology to identify differentially expressed genes
in children with sepsis (cases) when compared with


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children without an acute illness (controls). The eval-
uation of differentially expressed genes in a particular
illness, when compared with healthy controls, helps
in understanding the biological pathways and patho-
physiology of the disease processes. However, the
use of healthy controls in studies aiming to identify
gene expression markers that discriminate a partic-
ular disease from other illnesses may lead to spectrum
bias and fail to account for time-resolved differ-
ces. Further, the use of other modal data inclu-
sive of metabolites, in particular lipids, proteomic,
cellular and clinical observations allow for a more
complete picture of the underlying pathophysiology
and diagnostic algorithm development. We propose
a prospective cohort methodology to investigate the
differentially expressed genes over time in children
with sepsis and other acute illnesses.

METHODS
Hypothesis and specific aims
A neonatal sepsis classifier integrating immune and
metabolic pathways was able to discriminate neonates
with bacterial infections and sepsis with high accur-
cy.10 We hypothesise that the integrative tripartite
immune-metabolic pathways represented by differ-
entially expressed genes between sepsis and other
illnesses would discriminate bacterial infection causes
of sepsis from COVID-19 illness and other acute non-
infectious illnesses. The immune-metabolic pathways
vary between different age groups in children under
18 years. Our specific aims include:

► Compare immune and metabolic markers, which
discriminate sepsis and COVID-19 from other acute
non-infectious illnesses in children admitted to paedi-
atriac intensive care unit (PICU).

► Investigate the evolution of immune responses in
children with sepsis and COVID-19 using serial
samples.

► Evaluate the utility of gene expression markers
to discriminate heterogeneous subgroups of sepsis and
other critical illnesses with the help of deep clinical
phenotyping.

► Understand physiological processes associated with
COVID-19 and PIMS-TS and how they differ from
bacterial sepsis aetiology.

► Explore age-dependent differences in systemic host
response and determine whether these differences are
associated with the risk of disease severity to infection.

We plan to explore the feasibility of objectively
defining the balance between immune tolerance and
resistance in general, as well as in different paediatric
age groups. This is through investigating pathway
biology response to sepsis and multivariate analyses.

This is a prospective cohort study with a nested case-
control analysis. The start date was 30 June 2020 and the
proposed end date is 01 June 2023.

Inclusion criteria
Children or young people under 18 years who:
1. Are admitted to paediatric critical care unit (PCCU).
2. Have an acute illness including trauma.
3. Have routine blood tests as part of their clinical care.
   (or)
   Children or young people under 18 years who are
   admitted to paediatric wards with confirmed COVID-19
   illness or PIMS-TS.

Exclusion criteria
1. Admitted to hospital for social reasons without an
   acute illness.
2. Declined consent by parents or carers with legal re-
   sponsibility, or by competent young person.
3. Admitted to PCCU electively without an acute illness.
4. Direct clinical care team not able to provide research
   information in a language appropriate for non-
   English/Welsh speaking participants.

Patient/family journey
Any child or young person with an acute infectious illness
or a non-infectious illness such as trauma on admission
to PCCU will be eligible for participation in the study.
All children will be screened by the direct clinical care
team and research nurse. Research blood samples will
be collected in those who are eligible. Data collected
will include clinical and laboratory information essential
for clinical characterisation including clinical profile,
the severity of illness and clinical outcomes. There is no
follow-up planned nor repeat blood samples following
discharge from the hospital. Anonymous clinical data
during the index hospital admission will be collected
(figure 1).

Research blood sample collection
Research blood sample will be collected at admission or
onset of an acute illness in eligible children on PICU.
In COVID or PIMS-TS patients on paediatric wards and
who do not need intensive care, research blood samples
will be collected once consent is obtained. Further blood
samples may be collected but only alongside clinical
blood samples and at least 24 hours apart during the
illness, aiming to collect a final sample during recovery
from organ failure. All research blood samples will only
be collected along with blood samples required for
routine clinical care.

Case definitions and group stratification criteria
We aim to retrospectively carry out deep clinical phenotyping
of all patients recruited for the study using anonymous data
from the case report forms (CRF) (online supplemental
appendix 1). Two clinicians will review the data inde-
pendently to allocate a diagnosis to all recruited patients. The
allocation will be broadly into ‘infectious illness’, and ‘non-
infectious illness’, with further categorisation into various
specific illnesses including COVID-19 and sepsis (online
supplemental appendix 2 and 3—clinical phenotyping tool
and clinical phenotyping flow chart, respectively). In case
of any disagreement among the two clinicians, a third clinician will provide a final decision. This forms the reference standard for classification of illness, to aid interpretation of research sample analysis results.

The current practice is to isolate all children admitted to PCCU and wards until their SARS-CoV-2 PCR test result is known. Only those cases that meet the clinical phenotype of COVID-19 illness will be categorised as

Figure 1 Patient recruitment and blood sample analysis workflows. EDTA, Ethylene Diamine Tetra acetic Acid MS, mass spectrometry; PAXgene, Potassium Amyl Xanthate gene; PCCU, Paediatric Critical Care Unit; PIMS-TS, Paediatric Multisystem Inflammatory Syndrome Temporally related to SARS-CoV-2.
COVID-19. Patients who are coincidentally positive for SAR-CoV-2, but identified with other illnesses on clinical phenotyping, will not be categorised as COVID-19.

As the revisions to the definition of sepsis are pending, clinical phenotyping will be broadly based on the surviving sepsis consensus definition (2005). Patients will be included if they meet two or more criteria of SIRS, with at least one criterion related to white cell count or temperature, and with confirmed or strongly suspected to have an infection. There have been algorithms used previously to identify bacterial infections. In this study, we plan to evaluate sepsis due to any aetiology including fungal or viral infections. The clinical phenotyping will aid in categorising patients into descriptive clinical clusters, which we believe would have more utility in studying differences in immune-metabolic biological pathways between sepses due to viral, bacterial or fungal sepsis or where there is no microbiological agent identified. The process aims to report those cases as sepsis, which may deviate from the 2005 consensus definition, but have strongly suspected or confirmed infection and life-threatening organ dysfunction as described in the latest surviving sepsis consensus guidance statement. In addition, we can study the differences in sepsis as a whole in comparison with other acute illnesses. In those patients with confirmed infections and who do not meet the SIRS criteria for sepsis, deep clinical phenotyping will help us in evaluating the immune and metabolic markers based on patient outcomes such as severity of illness and organ dysfunction.

**Recruitment and consent**

Recruitment of eligible children admitted to PCCU meets the criteria for emergency research as we need to collect research blood samples soon after admission to the critical care unit, along with routine blood tests and it would not be inappropriate to approach parents or legal guardians for consent. We have ethics approval for research before consent and collection of multiple samples if the research team find parents not ready to make an informed choice to consent for research. Consultee advice will be sought in patients who are 16 and 17 years old who lack capacity or are not able to make an informed choice due to the critical illness.

In patients admitted to wards with confirmed COVID-19 or PIMS-TS, consent will be obtained after appropriate information sharing before research sample collection at the time of routine clinical blood sampling.

**Patient and public involvement**

The study group have engaged with representative members of public for the design of the study. Specific areas where this has led to incorporation of ideas and feedback are listed below:

1. Justification for the Study: Early identification of severe infectious illness will provide a diagnosis and information on prognosis will help in counselling and communicating with patients, families and parents.

2. Representatives of parents have approved of the methods involving research before consent as this is considered research in an emergency setting. The feedback stressed the importance of timing in the approach to parents of prospective research participants, to allow information sharing and consenting in such a way it causes minimum distress. We agreed that the timing for the approach should be based on the parent’s and patients’ ability to understand the research and not based on the duration from collection of first sample.

3. In view of the importance of appropriate timing for informed consent, it was also agreed that we could take further samples before consent. This was only in a situation where the direct clinical care team and research nurses felt that the parents may be emotionally not ready for approach or prospective research participant was severely unwell.

4. Representative members of the public were involved in drafting information sheets for parents, carers and older children. The final drafts were approved as appropriate for sharing information with parents and patients at the time of initial approach and consenting. Even though feedback about the information provided was incorporated, the information could not be shortened due to regulatory requirements.

5. Feedback from patient groups through Wales Gene Park included the research methods and information shared with families and legal guardians. Also included specific information related to next-generation sequencing in the study. The feedback was supportive of the methods used and suggestions were incorporated into the information sheets.

Peer review and feedback were sought through the UK Paediatric Intensive Care Society—Study Group as well as during a previous grant application.

**SAMPLE PROCESSING AND DATA ANALYSIS**

**Sample storage and analysis**

The blood samples, at clinical sites, will be stored at −20°C until transferred to the laboratory for processing, where they are stored at −80°C, once consent is obtained (figure 1).

Blood samples transferred to the laboratory will be processed in batches and initially retained by the pSeP team onsite at Cardiff University in a dedicated locked-secured freezer. For long-term storage and access for future research, samples will be housed by the Cardiff Biobank. For the transcriptomic and lipidomic analysis, the blood is mixed with the stabilising reagent potassium amyl xanthate in the collection tubes, all cells are immediately lysed and are not considered to be human tissue. The second tube will be stored as an Ethylene Diamine Tetra acetic Acid (EDTA) whole-blood sample (with or without a stabiliser for cellular phenotyping) and after cell, and targeted proteomics and metabolomics analyses will be banked for future use, for validating future diagnostic platforms.

We aim to apply a systems biology multiomic analysis of blood samples. This will involve using microarray and RNAseq methodology for probing the transcriptome of whole blood. While for specific immune cell characterisation, we will use techniques such as the Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-Seq) methods that perform RNAseq along with quantitative and qualitative information on surface proteins at a single-cell level. For metabolite and proteomic analyses, we will use the methods of liquid chromatography with tandem mass spectrometry (LC-MS/MS) in a targeted high-throughput manner, and we will use quantitative lipidomic profiling (LC-MS/MS) for comprehensive screening of specific pathways such as the complement system.

**Clinical data**

CRF will be used for collecting anonymous clinical data, including the clinical classifier (figure 1) from clinical phenotyping (online supplemental appendix 1).

**Sample size calculation for transcriptomic analysis using RNAseq**

We aim to recruit 250 patients with acute illnesses admitted to our PICU. Extrapolating the results of previous reports on the prevalence of severe sepsis in the paediatric intensive care population, we estimate that there will be around 25 patients with sepsis included in our study.\(^{32,33}\)

For calculating the power and type 1 error (\(\alpha\)), we refer by quality control and data processing using Bioconductor packages,\(^{35,36}\) before mapping to the human genome using Spliced Transcript Alignment to a Reference.\(^{37}\) Once we have read counts per gene/transcript, we plan to use R Language and Environment for Statistical Computing\(^{38}\) followed by quality control and data processing using Bioconductor packages.\(^{39}\)

A per-gene hypothesis of differential average expression will be tested using a negative binomial generalised linear model—DESeq2 package and resulting p values will be controlled for multiple testing using the Benjamini-Hochberg method.\(^{40}\) For classification, a variety of machine learning and statistical pathway biology approaches, as described in Smith et al.,\(^{41}\) will be used. Pathway analyses will be carried out stepwise using a pathway biology approach, becoming more focused. For metabolomic and proteomic data, absolute concentrations (determined by LC-MS/MS) of analytes in extracts from blood samples will apply validated tools such as MetaboAnalyst V.4.0.\(^{42}\)

Further multivariate statistical testing (Principal Component Analysis, Partial Least Squares Discriminant Analysis, Random Forest and Analysis of Variance) using group assignment derived from clinical phenotyping (types of sepsis and between sepsis and non-septic controls) will also be used to determine metabolites or proteins that are significantly different in abundance between clinical phenotypes. Single-cell analyses (CITE-Seq) using surface protein and RNA libraries (10\(\times\) Genomics) and next-generation sequencing will be multiplexed by cell ‘hashing’.\(^{43}\) Demultiplexed sequencing data will be aligned to the reference transcriptome using CellRanger (10\(\times\) Genomics) and the number of unique molecular identifiers per cell will be quantified. Computational analyses and quality control will be performed using R packages including Seurat to integrate hashtag, protein and RNA libraries while also enabling demultiplexing of donors, doublet detection and cell clustering.\(^{44}\)

For the final pathway biomarker assessment of the predictive success of the model, Receiver Operator Characteristics will be applied.

**DISCUSSION**

Through this prospective observational cohort study, we plan to evaluate immune-metabolic biological pathways in children with acute illnesses admitted for paediatric critical care. The aim is to evaluate the differences in immune and metabolic pathways in children with sepsis and other illnesses including COVID-19 in children and young people. The use of the cohort design will help in recruiting children of different age groups from birth to under 18 years admitted to PCCU with acute illnesses. The subsequent nested case–control analysis of different clinical groups from the cohort matched for age will help to identify differentially expressed genes among the different clinical phenotypes such as COVID-19 and sepsis with microbiological confirmation, COVID-19 and other viral infections, PIMS-TS and viral infections, PIMS-TS and sepsis with microbiological confirmation, sepsis with microbiological confirmation and confirmed viral infection. The initial analysis in a discovery cohort will be followed by cross-validation in a subsequent validation cohort. Feedback from parents and patient representatives has highlighted the importance of paediatric research for early identification of infections with subsequent poor outcomes or need for organ support. We hope that this study will help in planning future studies addressing this need. We plan to include children with polytrauma as this is considered an example of sterile inflammation.

**ETHICS AND DISSEMINATION**

Ethical approval was obtained from the Wales Research Ethics Committee 2 (IRAS project ID250612, REC ref: 20/YH/0214). Operational approval was received from...
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