Immunology of Thymectomy and Childhood Cardiac Transplant cohort study

Abbreviated study name: ITHACA

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Ethics reference: **21/NS/0142**

Signatures: The approved protocol should be signed by author(s) and/or person(s) authorised to sign the protocol.

SIGNATURE PAGE

The undersigned confirm that the following protocol has been agreed and accepted and that the Principal Investigator agrees to conduct the study in compliance with the approved protocol and will adhere to the principles outlined in the GCP guidelines, the Sponsor's (and any other relevant) SOPs, and other regulatory requirements as appropriate. Every care was taken in its drafting, but corrections or amendments may be necessary. These will be circulated to all relevant parties involved in the study.

I agree to ensure that the confidential information contained in this document will not be used for any other purpose other than the evaluation or conduct of the clinical investigation without appropriate authorisation.

I also confirm that I will make the findings of the study publicly available through publication or other dissemination tools, that an honest accurate and transparent account of the study will be given, and that any discrepancies and serious breaches of GCP from the study as planned in this protocol will be explained.

For and on behalf of the Study Sponsor:

Signature: ..... Date: ...../...../.....

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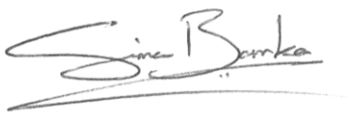
Principal Investigator:

Date: 07/07/2022

Signature: 

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ABBREVIATIONS AND DEFINITIONS

ACM – Acquired Cardiomyopathy

CHD – Congenital Heart Disease

CI – Confidence Interval

CLIA – Chemiluminescent Immunoassay

CMV - Cytomegalovirus

CRF – Case Report Form

eCRF – Electronic Case Report Form

EBNA – Epstein-Barr Virus Nuclear Antigen

EBV – Epstein-Barr Virus

EDTA – Ethylenediaminetetraacetic acid

GCP – Good Clinical Practice

GOSH – Great Ormond Street Children's Hospital

MHC – Major Histocompatibility Complex

NHS – National Health Service

NHSBT – NHS Blood and Organ Transplant

NJRO – Newcastle Joint Research Office

NuTH – Newcastle upon Tyne Hospitals NHS Foundation Trust

PBMCs – Peripheral Mononuclear Cells

PCR – Polymerase Chain Reaction

PTLD – Post-Transplant Lymphoproliferative Disease

SOP – Standard Operating Procedure

SOT – Solid Organ Transplant

SST – Serum Separating Tube

TIME – Tumour Immune Microenvironment

TRECs – T receptor excision circles

VCA – Viral Capsid Antigen

WHO – World Health Organisation

1. BACKGROUND AND RATIONALE

Post-transplant lymphoproliferative disease (PTLD) is a potentially fatal complication of childhood solid organ transplant (SOT), predominantly consisting of cancers that are histologically identical to sporadic childhood lymphomas. Recent work by our research group has shown that the 5-year cumulative incidence of post-transplant lymphomas following childhood heart transplant is amongst the highest, at 10% compared to approximately 5% in other childhood SOTs [1]. The driver(s) for this disparity are still poorly understood. PTLD has one of the worst outcomes amongst childhood lymphomas, with an Event-Free Survival of approximately 70% compared to 94% following a diagnosis of histologically identical sporadic B-cell lymphoma in the general population [2,3].

While PTLD is treatable, therapeutic complications from organ rejection and treatment toxicity present major clinical challenges. The clinical impact is particularly significant following childhood heart transplant due to the high incidence and mortality from organ rejection [4]. Risk factors for PTLD are complex and involve the interplay between Epstein-Barr virus (EBV) driven lymphoproliferation, iatrogenic immunosuppression and the suspected exhaustion of T-lymphocytes due to graft-initiated chronic antigen stimulation [5]. The role of EBV is clearly established in children, many of whom experience symptomatic primary infection from an EBV mismatched organ [1,2,6]. *In vitro & in vivo* studies focused mainly on adult monomorphic PTLD have demonstrated distinct patterns of viral protein expression in infected B-lymphocytes. [5,7] These expression patterns likely influence both the cellular composition of the tumour immune microenvironment (TIME) and aberrant immune signalling which result in immune escape of tumour cells.[5]

In our recent study examining risk factors for PTLD in the largest UK cohort of paediatric orthotopic heart transplant (OHT) patients to date (n=200), all 35/200 PTLD cases were EBV positive with PTLD risk being significantly higher in children with congenital heart disease (CHD) [Hazard Ratio=3.2; 95%CI=1.4–7.4] and early thymectomy at < 1 year old [HR=2.7; 95% CI=1.3–5.2] [1]. Furthermore, children with CHD had persistently lower T-lymphocytes than children transplanted for acquired cardiomyopathy [CD4+: 430/µl vs 963/µl, p< 0.01 and CD8+: 367/µl vs 765/µl, p< 0.01] [1].

Cardiac surgery via median sternotomy in early childhood routinely requires thymectomy in order to access the heart and great vessels. During the first year of life, the thymus plays a crucial role in the development of cell-mediated/adaptive immunity, providing a microenvironment for precursor T-lymphocytes to proliferate and differentiate into mature (naïve) T-lymphocytes [8]. This robust T-lymphocyte repertoire supported by a similarly healthy innate immune population is then more effective at controlling an EBV infection after primary

exposure. Whilst it has been shown that neonatal thymectomy alone is associated with premature immunosenescence and an increased risk of viral infections such as Cytomegalovirus (CMV) [9], little is known about the immunological consequences of early thymectomy in immunosuppressed transplant patients. In particular, there are no data on the impact of thymectomy/transplant on the immune response to EBV exposure including EBV-specific T-lymphocyte immunity and the subsequent risk of developing PTLD.

This study will investigate the development of EBV-specific immune responses following childhood heart transplant. Specifically, it will identify the impact of early thymectomy – compounded by iatrogenic immunosuppression – on EBV immunology and the risk of PTLD.

2. AIM AND OBJECTIVES

2.1 Aim

1) To investigate the systemic immune profile and EBV-specific immunity of early versus late/non-thymectomised transplant patients.

2.2 Primary Objectives

1) To dissect the peri-transplant innate and adaptive immune cell populations and their temporal responses to EBV infection in early versus late/non-thymectomised patients.
2) To define the peri-transplant EBV-specific immunity in early versus late/non-thymectomised patients.

2.3 Secondary Objectives

1) To detect peri-transplant immune signatures that either promote or inhibit PTLD development.
2) To identify potential biomarkers that can be used for patient risk stratification in future clinical trials of patients with lymphoproliferative disorders.

3. VALUE OF THE STUDY

This will be the first attempt to examine and sequentially monitor the immune response to EBV infection within a group of patients known to be at high risk of PTLD, while simultaneously exploring how this contributes to the disease process. The comprehensive assessment of EBV immunity in children with a heart transplant also provides a unique opportunity to fully understand the health impact of routine early thymectomy during major childhood heart surgery, a procedure whose consequence has never been explored within the transplant population. This study will address these clearly unmet needs of childhood cancer research by providing highly detailed immunological profiles of paediatric heart transplant patients, identifying systemic peri-transplant immune patterns associated with the risk of developing PTLD and potential mechanisms underlying the pathogenesis of PTLD. Furthermore, it will provide an in-depth understanding of the complex interplay between thymectomy, iatrogenic immunosuppression and impaired EBV-specific immunity and how these factors support an immune microenvironment that drives PTLD development. This will help to illuminate key differences between EBV-related PTLD in immunosuppressed patients and other EBV driven B-cell lymphomas in immune competent patients, thus leading to a better understanding of viral oncogenesis. The outcomes of this project are also vital to facilitate future patient risk stratification as well as the development of more effective treatments for PTLD.

4. STUDY DESIGN

4.1 Summary of Study Design

This prospective observational multicentre cohort study will recruit patients aged 0 – 18 years from the Newcastle and Great Ormond Street Hospitals, the two U.K. centres commissioned to provide paediatric heart transplant services. Thirty-four prospective cardiac transplant patients will be recruited over 2 years, based on a national average of 30 childhood cardiac transplants/year,[10] and a 60% recruitment rate. Peripheral blood samples will be collected immediately pre-transplant and at 3, 6, 12 and 24-months post-transplant (Figure 1). A non-thymectomy control cohort will consist of 6 age-matched renal transplant recipients. Study cohorts will be stratified to early thymectomy (<1 year of age) and late (>1 year of age)/non-thymectomy and used to fulfil the study objectives. Blood samples for evaluation of EBV serology will be tested in NHS microbiology/virology laboratories in the transplanting centre and circulating immune cell populations will be analysed in Newcastle University laboratories. Clinical data will be collected locally using a purpose-built electronic case report form (eCRF).

Immunosuppressive therapy will follow standard local protocols and will be adjusted according to clinical needs as per those local protocols. Any deviations from the protocol will be documented in the eCRF. Children will undergo blood sampling for study specific investigations during routine clinical visits. No additional hospital visits are planned. Medical therapy will be applied as clinically indicated and per the local post-transplantation protocols. No additional interventions are proposed by the ITHACA study protocol.

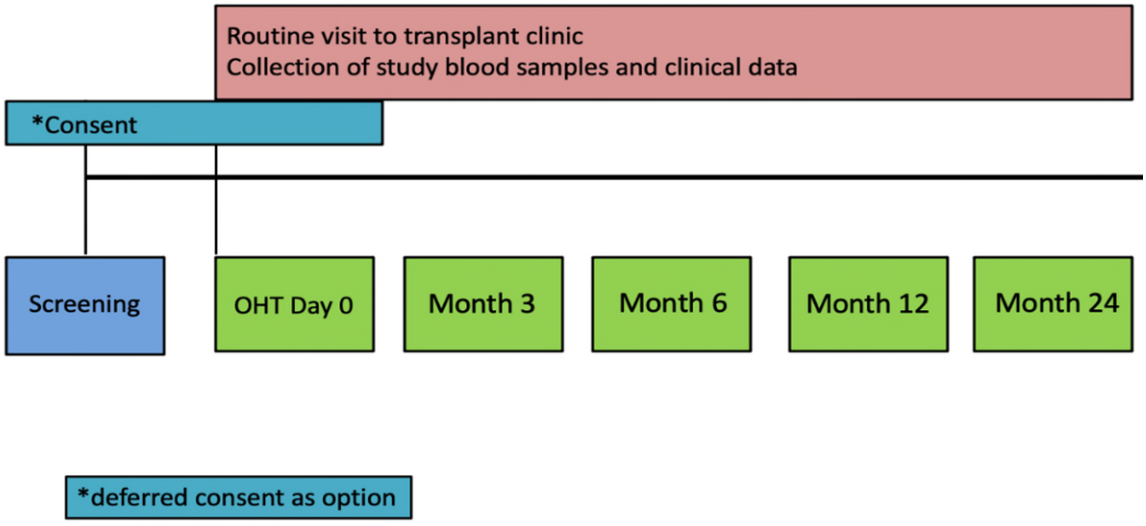

Figure 1: ITHACA study flow chart. **OHT** – Orthotopic heart transplant

4.2 Primary and Secondary Endpoints/Outcome Measures

Primary Outcomes Measures:

1) Proportions of circulating innate and adaptive immune cell subsets immediately before and at 3-, 6-, 12- and 24-months post-transplant. [Time frame: 2 years]
2) Frequency of detectable EBV-specific T-lymphocyte immunity. [Time frame: 2 years]

Secondary Outcome Measures:

1) Incidence of EBV infection. [Time frame: 2 years]
2) Time from transplantation to EBV viraemia. [Time frame: 2 years]
3) Time from EBV viraemia to seroconversion. [Time frame: 2 years]

4.3. Study Participants

4.3.1. Overall Description of Study Participants

Children and young people who have recently undergone or are about to receive a cardiac or renal transplant will be eligible for this study.

4.3.2. Inclusion Criteria

- Resident in the UK.
- Aged 0 – 18 years.
- Actively listed on the NHS Blood and Transplant (NHSBT) waiting list for a primary organ transplant OR awaiting transplant with a living related donor kidney OR recently transplanted with pre-transplant blood samples available.
- Written informed consent.

4.3.3. Exclusion Criteria

The participant may not enter the study if ANY of the following apply:

- Has a pre-existing diagnosis of an inherited or acquired immunodeficiency.
- Has an underlying thymic disorder.
- Has previously received a bone marrow or organ transplant.
- Has had a previous cancer diagnosis.
- Withheld consent.
- Weight under 2.5kg (see section 3.4.6).

4.3.4. Withdrawal Criteria

Due to the observational nature of the study and negligible risk to the health of study participants, withdrawal should occur only upon patient and/or parental request.

4.4. Study Procedures

4.4.1. Summary of tests

An extra 5 – 20mls of blood will be taken at the time of the patient's usual investigations in adherence to WHO guidance for blood sampling in child health research (See section 3.4.6). Study blood samples will consist of:

Laboratory test	Weight 2.5kg – 20kg	Weight > 20kg
EBV PCR	1 x 1ml K2 EDTA tube	1 x 3ml K2 EDTA tube
EBV VCA IgM/IgG/EBNA1	1 x 1ml SST™ GEL tube	1 x 4ml SST™ GEL tube
TRECs	1 x 1ml K2 EDTA tube	1 x 3ml K2 EDTA tube
PBMCs for innate/adaptive immune cell markers	2 x 1ml K2 EDTA tube	2 x 5ml K2 EDTA tube

Table 1: Study blood samples and volumes required according to participant body weight (kg).

Clinical information to be collected for all study participants are listed in Table 2.

Category	Clinical data
I. Baseline patient details	Age at transplant, Age at thymectomy*, Cardiothoracic procedure at time of thymectomy, Sex, Ethnicity, Weight, Height, Comorbidities prior to transplantation, Concomitant medication at time of transplantation.
II. Transplant-related	Cardiac/Renal diagnosis, Indication for transplant, Blood group compatibility.
III. Infection-related	EBV and CMV (where available) serostatus of donor & recipient at transplant.
IV. Immunosuppression	Induction therapy, maintenance immunosuppression drugs (dose and trough levels at each follow up visit), changes to maintenance immunosuppression drugs and indication for changes.
V. Complications	Opportunistic infections, Graft failure/rejection, PTLD, Mortality, documentation of "other" complications.
VI. Follow up	Weight, Height, Additional medication, Relevant additional comments (also see categories IV & V)

Table 2: Clinical data for ITHACA Study. *****Age at 1st median sternotomy is an agreed proxy where thymectomy isn't clearly documented in the patient's surgical notes.

4.4.2 Identification of patients

Potential participants will be identified when they present to the paediatric transplant services at Newcastle upon Tyne NHS Foundation Trust Hospitals (NuTH) or Great Ormond Street Children's Hospital (GOSH) as nationally listed candidates for urgent cardiac or renal transplantation.

All patients who meet the study inclusion criteria will be deemed eligible and approached regarding participation in the study by members of either the local transplant or study teams. If the patient/carer is non-English speaking then a translator will be present at this meeting for a face-to-face conversation, if the patient/carer approves.

At the GOSH research site, either the research nurse or a member of the clinical transplant team will approach the family and obtain consent for the study.

4.4.3. Informed Consent

The study specific Participant Information Sheet (PIS) will be made available to participants prior to consent being obtained. An initial screening consent will be obtained from the parent/carer of the eligible child or from the patient themselves if over the age of 16 years. The screening consent will permit the recruitment of all eligible children on the transplant waiting list, who may proceed to transplant during the study period. It will also give study permission for the pre-transplant study sample to be taken and stored. Additional informed consent will be required for any child who then progresses to receive their transplant after providing the initial screening consent. This informed consent will give permission for formal recruitment to the study, analysis of blood samples, and collection of clinical data. Assent may be given by children < 16 years of age who wish to do so.

A deferred consent approach will be employed for potential study participants who attend a research site's transplant service for transplantation in a critical/life threatening clinical situation. This will involve the collection of baseline study samples from potential participants at the point of pre-surgical workup without written screening or informed consent being received. Discussion about the study, the giving of PIS and receiving of written screening consent/informed consent/assent will be offered at a more appropriate time before further follow-up blood tests are taken. Such cases will require that the clinical team considers obtaining informed consent prior to transplant to be inappropriate. This should be documented in the patient's clinical notes. Study samples collected under such circumstances will be processed for storage but not analysed until written informed consent is obtained. Any patient who has study samples collected by deferred consent but subsequently declines enrolment in

the study will have their samples destroyed in a timely manner according to local laboratory standard operating procedures (SOP).

At the NuTH site, the paediatric oncology research registrar - (U. Offor - Clinical Fellow), the chief investigator (S. Bomken), research nursing team or a trained member of the transplant clinical team, will explain the purpose of the study to the patient/carer and provide opportunity for questions and further discussion as necessary. A similar process will be followed by the study team at GOSH. Key points to reiterate to the patient/care will include:

- Participation will not affect the child's treatment and will not benefit them/their child at present but could benefit other children in the future.
- Participation in the study is voluntary and the volunteer can withdraw at any time without any explanation being necessary.

The parents/carer will be given clear guidance on how to contact the research team. They will also be informed that if consent is obtained, any remaining blood sample would be stored in the laboratory and added to the Newcastle Biobank at the end of the study. These samples could then potentially be used for future ethically approved research. A GCP trained member of the transplant or study research team will be responsible for obtaining informed consent at both research sites.

4.4.4. Timing of sample collection, processing and shipping

- On the day of their routine follow up assessment, patients will attend the transplant service's outpatient clinic (see Figure 1).
- They will be assessed by the clinical team to check they are safe to undergo the procedure.
- At the same time as their routine blood samples are being taken, an extra 5mls – 20mls of blood will be taken for this study and placed into BD Microtainer® tubes (see Appendix for further details).
- The study samples will be labeled with the patient's study number, time and date and bagged with the relevant completed sample form (see Appendix).
- Samples obtained in Newcastle will be collected by the Clinical Fellow (U. Offor) or Research Nurse to be taken to the Wolfson Childhood Cancer Research Centre, Newcastle University.
- In the case of samples collected at the GOSH site, the research nurse at this site will inform the Clinical Fellow (U. Offor) and/or the Chief Investigator (S. Bomken) via phone and/or email that a sample is being sent within 24 hours of being taken.

- GOSH samples will be packaged and sent via Royal Mail Special Delivery SafeBox at room temperature to the study address given in the Appendix.

4.4.5. Analysis of samples

The study samples for will be taken to the Translational and Clinical Research Institute, Newcastle University, where they will be analysed by the following methods.

1) Detection of circulating innate and adaptive immune cell populations using the Aurora spectral flow cytometer.
2) Detection of T Receptor Excision Circles (TRECs) as a marker of recent thymic output using flow cytometry.
3) Evaluation of EBV-specific CD4+ and CD8+ T-lymphocytes by MHC I/II tetrameric assay using flow cytometry.

In parallel, samples for EBV serology will be sent to the NuTH/GOSH blood sciences laboratories, where they will be analysed for EBV VCA IgM, IgG, and EBNA-1 antibodies using chemiluminescent immunoassay (CLIA) along with EBV viral copy quantification by PCR.

4.4.6. Potential adverse events

Blood samples - samples will be taken at the same time as routine transplant investigations, thereby avoiding any additional discomfort. The size of the child will determine whether the blood volume required can safely be taken without any risk of causing anaemia. Recent guidance from the World Health Organisation (WHO) for trial related blood volumes to be taken in children suggests that there is minimal risk with sampling of up to 5% of a child's total blood volume but that this could be reduced to 3% in the presence of serious illness.[11] Assuming a child's circulating blood volume is 80ml/kg (this is age dependent and is generally taken to vary from 80-90ml/kg for children), the safe upper limit of 3% of the total blood volume is 2.4ml/kg, which for a child of 2.5kg = 6mls. Since a minimum of 5ml blood is required for this study, we will only include children above 2.5kg in this study.

4.5 Time Frame

- Proposal completed Jan 2021
- Applications completed Jun 2021
- Data collecting commences Sept 2021
- Data collection complete Jan 2024
- Analysis complete April 2024
- Writing up completed Aug 2024

5. STATISTICS

5.1. Sample size

As the study objectives are largely descriptive, no sample size calculation is necessary. All patients listed on the national transplant register will be approached for screening consent. We expect to formally recruit 40 patients to be studied across the two U.K. centres commissioned to perform paediatric heart transplants (NuTH and GOSH). This cohort will consist of 34 prospective cardiac transplant patients and an age-matched control group of 6 renal transplant patients (see section 3.1).

5.2. Data analysis

Automated clustering of immune cell populations from spectral flow cytometry will be performed in R using FlowSOM and ConsensusClusterPlus packages (Bioconductor). Flow cytometry data will be analysed using FlowJo (Treestar). Statistically different immune signatures between patients with early thymectomy and late/non-thymectomy, EBV+ and EBV- serostatus, and between time-points, will be identified by linear and generalised linear mixed models (statistical significance defined as $p < 0.05$). Continuous variables will be assessed by Pearson correlation using the single linkage method to group patients by expression values, and non-continuous variables by non-parametric Spearman correlation, as appropriate. Viral loads will be serially quantified at each study timepoint to correlate changes in immune responses with the volume of circulating virus-infected cells. Tetrameric frequencies for population groups (e.g., early vs late/non-thymectomy) will be compared using Mann-Whitney U test, and between time-points for paired patient sample using Wilcoxon signed-rank test.

6. ETHICAL CONSIDERATIONS

6.1. Participant Confidentiality

Every effort will be made to maintain participant privacy. Clinical information will be entered by clinical or NHS research teams using bespoke eCRFs provided through an online data collection system, REDCap. REDCap is hosted on servers with security exceeding NHS specifications and is governed and managed by a dedicated IT team at NuTH. Minimal patient identifiable information will be collected about study participants, allowing validation of data across time points. Only chief and co-investigators will have access to data across all patients. Once data collection has been completed and audited to ensure accuracy, anonymised data will be exported from REDCap for ongoing analysis. A key linking patient identifiers and study ID numbers will then only be held by the chief investigator on a password protected, secure NHS server.

Patient samples will be identified using only the study identification number. No patient identifiers will be made available to non-NHS staff or in any submission or research documentation.

6.2. Patient and Public Involvement

The Young Person's Advisory Group North England (YPAGne) was involved in the development of the study design and informed consent process. Ongoing consultation with YPAGne will continue to influence participant recruitment, outcome measure priorities and the acceptability of study methods.

7. DATA MANAGEMENT AND DATA HANDLING GUIDELINES

All study data will be collected from sites on eCRFs using the REDCap system. The eCRF must be completed by staff who are listed on the site staff delegation log and authorised by the local Principal Investigator to perform this duty. Each authorised staff member will have their own unique login details for the eCRF. They must never be shared among staff as the eCRF audit trail will record all entries/changes made by each user. The local Principal Investigator is responsible for the accuracy of all data reported in the eCRF. The use of abbreviations and acronyms should be avoided.

Data must be accurately entered into the eCRFs and must be verifiable from source documents at site. Examples of source documents include hospital records such as patient notes, laboratory and other clinical reports, etc. The relevant eCRF forms must be completed as soon as possible after a patient's visit.

8. FINANCING AND INSURANCE

This study is funded by Cancer Research UK and The Lymphoma Research Trust. Insurance for the study design is provided by Newcastle University and NHS indemnity for study management.

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11. APPENDIX

Please contact U. Offor on 0191- & ugo.offor@newcastle.ac.uk **and** S. Bomken on s.n.bomken@newcastle.ac.uk to inform of a sample being sent.

Send samples in BD Microtainer® tubes, appropriately packaged by Royal Mail Special Delivery SafeBox at room temperature to:

Dr Ugo Offor & Dr Simon Bomken,
ITHACA study,
Wolfson Childhood Cancer Research Centre,
Level 6, Herschel building,
Newcastle University,
Newcastle-upon-Tyne,
NE1 7RU
U.K.

For supply of study documentation, BD Microtainer® tubes, and central data management please contact U. Offor or S. Bomken using the above contact details.

Immunology of THymectomy And Childhood CArdiac Transplant

Sample Form

v2.0, 24/06/2021

Site (please circle)	**NuTH Cardiology** **NuTH renal** **GOSH**
Study ID number	**_ _ / _ _**
Timepoint*	
Is informed written consent obtained?	**Yes / No**
Date taken	
Time taken	
Date received (NU lab use only)	
Time received (NU lab use only)	

*Timepoints – pre-transplant, 3 months, 6 months, 12 months, 24 months