This protocol has regard for the HRA guidance but not the order of content

Drug-induced Cardiotoxicity in Cancer Therapy

STUDY PROTOCOL

Translational insights into the underlying pathogenesis of anthracycline-induced cardiotoxicity

Version 2.1 May 17th 2022

MAIN SPONSOR: University of Liverpool

FUNDER: University of Liverpool

IRAS ID: 285910

Study Team

Chief Investigator: Dr Parveen Sharma

Clinical Investigators: Professor Jay Wright, Dr Rebecca Dobson, Professor Carlo Palmieri, Dr David Gent

SIGNATURE PAGE

The undersigned confirm that the following protocol has been agreed and accepted and that the Chief Investigator agrees to conduct the study in compliance with the approved protocol and will adhere to the principles outlined in the Declaration of Helsinki, the Sponsor's SOPs, and other regulatory requirement.

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 investigation without the prior written consent of the Sponsor

I also confirm that I will make the findings of the study publicly available through publication or other dissemination tools without any unnecessary delay and that an honest accurate and transparent account of the study will be given; and that any discrepancies from the study as planned in this protocol will be explained.

For and on behalf of the Study Sponsor:

Signature:
.....

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

Name (please print):
.....

Position:
.....

Chief Investigator:

Signature:
.....

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

Name: (please print): DR PARVEEN SHARMA
.....

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CLINICAL QUERIES

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STUDY SUMMARY

Long Study Title	Translation insights into the underlying pathogenesis of anthracycline-induced cardiotoxicity
Internal ref. no. (or short title)	Drug-induced cardiotoxicity in cancer therapy
Study Design	Multi-centre case control study
Study Participants	Chemotherapy patients with and without cardiotoxicity
Planned Size of Sample	12
Follow up duration (if applicable)	N/A
Planned Study Period	2 years
Research Aim(s)	The study's aim is to generate cardiomyocytes from patient-derived induced pluripotent stem cells and characterise the mechanisms of anthracycline-induced cardiotoxicity. This will be done bycomparing the electrical, structural, and functional differences between cells derived from patients exhibiting cardiotoxicity because of chemotherapy treatment compared with those on a matched treatment regime with no evidence of cardiotoxicity.

This protocol describes the 'Drug-induced Cardiotoxicity in Cancer Therapy' Study and provides information about procedures for entering participants. Every care was taken in its drafting, but corrections or amendments may be necessary. These will be circulated to investigators in the Study. Problems relating to this Study should be referred, in the first instance, to the Chief Investigator.

This study will adhere to the principles outlined in the UK Policy Framework for Health and Social Care Research (v3.2 10th October 2017). It will be conducted in compliance with the protocol, the EU General Data Protection Regulation 2016 and Data Protection Act 2018 , and other regulatory requirements as appropriate.

ROLE OF STUDY SPONSOR AND FUNDER

The Sponsor for this study is the University of Liverpool. The Funder for this study is the research fund at the Liverpool Heart and Chest Hospital. The Sponsor will take responsibility for the initiation, management, and arrangement of finance for the study. The Sponsor will not have any role in the study design, conduct, data analysis and interpretation, or manuscript writing and will not control the final decision regarding any of these aspects of the study. The Sponsor/Funder will be involved in the dissemination of the results of the study.

ROLES AND RESPONSIBILITIES OF STUDY MANAGEMENT COMMITEES/GROUPS & INDIVIDUALS

The Chief Investigator (CI) and Principal Investigator (PI) for this study is Dr Parveen Sharma, senior lecturer at the University of Liverpool (UoL). As the CI, Dr Sharma will be responsible for the conduct of the whole project and will communicate with the Research Ethics Committee (REC) and other review bodies during the application process and where necessary during the conduct of the research. As the PI, Dr Sharma will also be responsible for the conduct of the research at the University of Liverpool study research site. The study will have four additional clinical Co-investigators - Professor Jay Wright, consultant cardiologist at Liverpool Heart and Chest Hospital (LHCH) Dr Rebecca Dobson, consultant cardiologist at LHCH; Professor Carlo Palmieri, consultant oncologist at the Clatterbridge Cancer Centre (CCC) and the Royal Liverpool and Broadgreen University Hospital NHS Trust (RLBUHT); Dr David Gent, Cardio-oncology research fellow and cardiology registrar at the LHCH and PhD student at the University of Liverpool. The study's Co-investigators will lead the recruitment, study coordination, and conduct at their respective NHS Hospital Trust sites. As this is a pilot study, there will be no study steering group.

The **Study Management Team** consists of members of the research and clinical teams who will liaise regularly to refine the study plans and review its progress. Members of the Study Management Team are:

Dr Parveen Sharma – UoL/ CI/ PI
Dr Richard Rainbow – UoL/ Co-applicant
Professor Jay Wright – LHCH/ Co-investigator
Dr Rebecca Dobson – LHCH/ Co-investigator
Professor Carlo Palmieri – CCC/RLBUHT/ Co-investigator
Professor Gregory Lip – Liverpool Centre for Cardiovascular Science (LCCS) Director/ LHCH/UoL/

The conceptualisation of the project and details of the initial research plan were developed through dialogue between the study's **Co-applicants**:

Dr Parveen Sharma – UoL/ Co-lead applicant
Profession Jay Wright – LHCH/ Co-lead applicant
Dr Mike Cross – UoL
Professor Gregory Lip – LHCH/ LCCS Director
Dr Richard Rainbow – UoL
Professor Sir Munir Pirmohamed – UoL
Professor Chris Denning – University of Nottingham

PROTOCOL CONTRIBUTORS

This protocol was developed during the process of applying for funding from the University of Liverpool and was written with contributions from the following people:

Dr Parveen Sharma – University of Liverpool/ PI/ CI

Miss Laura Scott – University of Liverpool/ Research Assistant

Dr Richard Rainbow – UoL/ Co-applicant

Professor Jay Wright – LHCH/ Co-investigator

Dr Rebecca Dobson – LHCH/ Co-investigator

Professor Carlo Palmieri – CCC/RLBUHT/ Co-investigator

Professor Gregory Lip – LHCH/ LCCS Director/ Co-applicant

Dr David Gent – LHCH/LCCS/University of Liverpool Co-investigator

There was no patient or public involvement or contribution for any aspect of the protocol design.

KEY WORDS: Anthracycline, cancer, cardiomyocyte, cardiotoxicity, differentiation, doxorubicin, pluripotent, reprogramming, stem cell, transcription factor

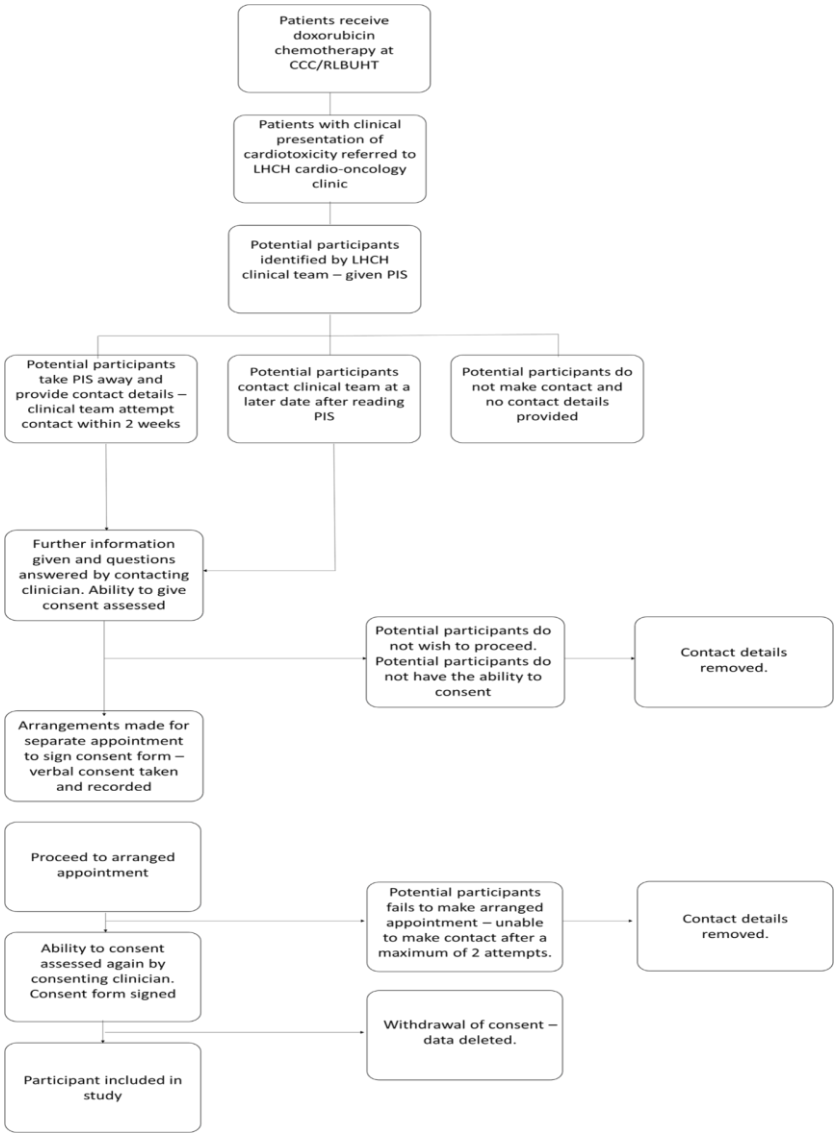
GLOSSARY OF ABBREVIATIONS

CCC	Clatterbridge Cancer Centre
CF	Consent Form
CI	Chief Investigator
CP	Carlo Palmieri (Prof)
CVD	Cardiovascular Disease
ESC	Embryonic Stem Cell(s)
GCP	Good Clinical Practice
GL	Gregory Lip (Prof)
HRA	Health Research Authority

HTA	Human Tissue Act and/or Authority
IPSC	Induced Pluripotent Stem Cell

IRAS	Integrated Research Application System
JW	Jay Wright (Prof)
LCCS	Liverpool Centre for Cardiovascular Science
LHCH	Liverpool Heart and Chest Hospital
LVEF	Left Ventricular Ejection Fraction
LVSD	Left Ventricular Systolic Dysfunction
PIS	Patient Information Sheet
PBMC	Peripheral Blood Mononuclear Cell(s)
REC	Research Ethics Committee
RD	Rebecca Dobson (Dr)
RLBUHT	Royal Liverpool & Broadgreen NHS Hospitals Trust
UoL	University of Liverpool
UoN	University of Nottingham

STUDY FLOW CHART



CORONAVIRUS (COVID-19) GUIDANCE

To account for the 2019-2020 SAR-COV-2 virus outbreak and the resultant COVID-19 disease pandemic the following considerations relevant to the study are in place and will remain in place for as long as government advice and/or the participating site dictates.

At the time of submission, the details contained below are correct and up to date. Any significant changes to site-specific COVID-19 policies will be added in an amendment and all users of this manual are advised to check the details with a senior member of staff if they are unsure.

For participating NHS hospital sites, all patients are asked to attend their appointments alone (no relatives allowed). The waiting room will be COVID-19 secure with appropriate social distancing in place. All staff within the clinic and hospital will wear appropriate PPE. All patients will have their temperature taken prior to being allowed into the department.

Research staff who collect the samples from the hospital will have no contact with participating patients to avoid unnecessary exposure. All research staff at the University of Liverpool research site will work under a purpose-based COVID-19 risk assessment until further notice and room access is restricted to an online booking system to maintain adequate social distancing and prevent over-crowding. Social distancing is maintained and appropriate PPE (including face masks) is worn by staff.

Guidance is reviewed and updated regularly, and participating sites and personnel will keep themselves informed and up to date on the latest procedures.

Details of the latest government guidance can be found at www.gov.uk/government/collections/coronavirus-covid-19-list-of-guidance

The most up to date advice and guidance regarding the management of COVID-19 in research studies, including that for sponsors, participating sites and researchers, is given at the following HRA web address https://www.hra.nhs.uk/covid-19-research/covid-19-guidance-sponsors-sites-and-researchers/#patients

NB, section 4.3 [Page..] of the study Protocol includes the following exclusion criterion: Active or recent serious infection as determined by the consenting clinician. This criterion will also take into consideration a potential active or recent infection with Covid-19.

1 INTRODUCTION

1.1. BACKGROUND

Anthracyclines are amongst the most widely prescribed and effective anticancer drugs in use, and remain an important part of treatment regimes in both adult and paediatric oncology practice [1-5]. The efficacy of anthracyclines however is hindered by the significant side effects that they induce. Cancer treatment is therefore no longer aimed exclusively at treating the malignancy, but also at the early identification and treatment of the toxic side effects. One of the potentially life-threatening toxic side effects of anthracycline use is cardiac toxicity [6, 7]. The cardiotoxic potential of anthracyclines routinely used in the treatment of breast cancer and haematological malignancies for example, such as doxorubicin, specifically requires close monitoring of cardiac function [8, 9]. Doxorubicin, the most commonly prescribed anthracycline [10] is known to cause potentially irreversible cardiomyocyte death, either through necrosis or apoptosis [5]. The exact cytotoxic mechanism of anthracyclines remain unclear, with contradictory theories observed within the literature [11-21].

The severity of side effects from anthracyclines such as doxorubicin is largely dose dependent, with cumulative administered dose being the most critical factor in the development of cardiotoxicity [22]. Anthracycline-induced cardiotoxicity typically manifests as left ventricular systolic dysfunction (LVSD) and congestive heart failure [23-25] with additional risk factors for prediction including age (< 4 years and > 60 years), significant hypertension, and female gender [9, 26]. In adults, the incidence of heart failure induced by doxorubicin varies from 4% to 5% at a cumulative dose of 500-550 mg/m2, to 36% at a cumulative dose of 600 mg/m2 or more [9, 22]. The cardiotoxic side effect of anthracyclines determines their cumulative maximal tolerable dose.

1.1.1. Categorising Cardiotoxicity

Anthracycline-induced cardiotoxicity is currently recognised clinically in three categories: acute, early-onset chronic, and late-onset chronic [27]. Acute toxicity is directly associated with drug infusion and typically resolves spontaneously within hours [28]. Whilst acute cardiotoxicity does not represent a serious clinical problem, chronic toxicity does. Early-onset chronic anthracycline cardiotoxicity is typically observed within weeks to months of the patient receiving their final treatment, whereas late-onset chronic cardiotoxicity sees a delay period that can last up to twenty years before evidence of cardiotoxicity eventually presents. Late-onset cardiotoxicity is often a complication observed in childhood cancer survivors [29, 30]. Acute cardiotoxicity has been shown to occur in less than 1% of patients immediately after anthracycline administration whilst early-onset chronic cardiotoxicity occurs in 1.6 - 2.1% of patients within one year of therapy (with a peak incidence at three months post treatment) and late-onset chronic cardiotoxicity occurring in approximately 5% of patients more than one year post therapy [31].

1.1.2. Prevention and management of cardiotoxicity

Primary and secondary prevention strategies in the management of anthracycline-induced cardiotoxicity involve preventing cardiac damage at the time of chemotherapy treatment (primary) and preventing the progression to symptomatic disease following the detection of LVSD (secondary)

[32]. No evidence-based guidelines currently exist for monitoring cardiotoxicity during and after anticancer therapy and published expert consensus guidelines remain inconsistent [33-37] .Whilst some cardioprotectants have shown to markedly suppresses anthracycline cardiotoxicity, their limited specificities are unable to interfere with all of the proposed potential mechanisms of cardiotoxicity [38-40]. Characterising the pathophysiology of its development and progression is therefore crucial to designing more protective treatment strategies and identifying the most appropriate monitoring techniques.

1.1.3. Clinical monitoring of cardiotoxicity

The main goal of cardiotoxicity detection is to predict as early as possible those at risk of developing heart failure before they develop irreversible dysfunction. Evaluation of cardiac function is considered essential [41], but effectively doing so remains a challenging task, with current methods relying on either diagnostic imaging alone or a multi-modality approach of diagnostic imaging and blood-based biomarker analysis.

Echocardiographic strain imaging is the standard method in clinical practice for evaluating cardiac function and has shown great value in the detection of cardiotoxicity. Echocardiography is used to assess left ventricular ejection fraction (LVEF) which measures global left ventricular (LV) volumetric change. Once LVEF is significantly reduced, restoration of normal function is possible but can sometimes be difficult [42].

Blood serum biomarkers have also shown to be useful in the early identification of anthracycline-related cardiotoxicity [43]. Early and persistent elevation of cardiac troponin (the biomarker of choice for identifying myocardial injury) has been shown to identify patients who are more likely to develop symptomatic heart failure and benefit from supportive therapies [42]. In early studies, elevations of troponin T (TnT) levels were recorded following initial therapy with doxorubicin, with the magnitude of elevation predicting LV thickness and wall thinning nine months later [44]. Elevated troponin I (TnI) levels have been documented in anthracycline-treated patients compared to healthy controls and anthracycline-naive patients [45], whilst Kilickap et al showed an increase in TnT in 41 patients treated with anthracycline-based chemotherapy [46].

Another marker used in the diagnosis and assessment of heart failure is the 32-amino acid polypeptide - B-type natriuretic peptide (BNP) and its N-terminal fragment (NT-proBNP). The synthesis of BNP occurs in the ventricles of the heart and serum levels correlate with the severity of heart failure and ventricular pressure. Both markers have proven their diagnostic usefulness in several studies and have since progressed to clinical application [47-49].

A number of approaches aimed at minimising anthracycline-induced cardiotoxicity have also been used in clinical practice, such as tailoring the cumulative dose, altering the treatment schedule, and prescribing cardioprotective agents to act against one or more of the damaging effects associated with anthracycline use.

1.1.4. Proposed study

Anthracycline-induced cardiotoxicity is a clinical burden that has a significant impact on the wellbeing and life expectancy of cancer patients. Characterising the pathophysiology of its development and

progression is therefore crucial to designing a successful protective treatment strategy and identifying the most appropriate monitoring techniques. In this study we want to characterise the development and progression of anthracycline-induced cardiotoxicity through proteomics profiling and functional experimental assays. To achieve this, we will generate patient-derived cardiomyocytes using a technology that has revolutionised cardiovascular research in the last decade. Instead of seeking to obtain an invasive biopsy sample from participants, we will employ stem cell technology to produce large quantities of stem cells through integration-free viral transduction and/or transfection using only a small blood sample. From there, we will develop cardiomyocytes that recapitulate the donor phenotype. The sample size will be small and will include cancer patients who have developed cardiotoxicity as a result of receiving anthracyclines during their chemotherapy treatment regime. We will screen these samples against age-matched chemotherapy patients who have received the same treatment regime but have not developed cardiotoxicity.

1.2. RATIONALE FOR CURRENT STUDY

According to the World Health Organisation, cardiovascular diseases (CVD) are the number one cause of death worldwide, with almost 23.6 million people estimated to die from CVD by 2030.The second leading cause of death globally is cancer, with 9.6 million people estimated to have died from the disease in 2018. The greatest single non-cancer cause of death in cancer survivors is CVD.

Cancer Research UK states that the cancer survival rate in the UK has doubled in the last 40 years from 24% to 50% at 10 years in many of the most commonly diagnosed cancers. This can be attributed to improvements in the understanding of the cause and management of the disease as well as the availability of new and evolving treatment options.

One of the most prominent and effective forms of cancer treatment to date has been the use of anthracyclines, which are known to cause cardiotoxicity in patients both during and several years following treatment. This is also true in teenagers and young adults who receive anthracycline therapy during childhood. The dose-dependent cardiotoxic effects of chemotherapeutic anthracyclines can limit patient exposure and therapeutic efficacy. Even at relative low cumulative doses, an 8% increase in adverse cardiac events has been observed which increases to 26% with cumulative doses and adjuvant therapy with targeted monoclonal antibodies [50]. Whilst improvements have been made into understanding the cause and management of cancer over recent years, the same level of understanding has not yet been reached into the cause and management of cancer-related comorbidities like anthracyclines-induced CVD.

With cancer survival statistics on the rise, recent consultation with oncologists and cardiologists from the Liverpool area highlights the fact that attention is now switching to the need for more effective monitoring and management of cancer patients for CVD following treatment. The clinical management of such complications however currently lacks scientific support. To effectively aid this, better understanding is warranted to explain why some cancer patients are at a higher risk of developing and dying from CVD compared to others. CVD risk in patients receiving anthracycline therapy has been shown to correlate with several clinical and lifestyle factors, such as age, sex and smoking, but being able to stratify baseline risk in patient populations on additional histological and genetic factors would greatly advance and improve supportive therapeutic approaches. This study

aims to achieve an insight into what the histological and genetic factors might be and whether they have any predictive or prognostic value.

The primary aim of this study is to generate cardiomyocytes from patient-derived induced pluripotent stem cells (iPSC) and characterise the mechanisms of anthracycline-induced cardiotoxicity by comparing the electrical, structural and functional differences between cells derived from patients exhibiting cardiotoxicity as a result of chemotherapy treatment compared with those on a matched treatment regime with no evidence of cardiotoxicity. Studies have shown that patient derived iPSC cardiomyocytes recapitulate the characteristics seen in the patients from whom they are derived, and since the heart is a non-regenerative organ the availability of biopsies is rare, particularly from healthy volunteers, therefore limiting the analysis and comparisons from the primary source.

The incidence of anthracycline-induced cardiotoxicity in the adult population is difficult to determine as follow-up time and monitoring policies are often currently inadequate. Due to the limited underpinning of scientific judgement on clinical management in these patients, current recommendations are based on expert consensus and local multidisciplinary protocols.

This study should help to identify candidate risk factors for anthracycline-induced cardiotoxicity on a physiological level that will provide a more specific prediction model than that which currently exists, thus aiding in improving both life expectancy and quality of life following cancer treatment.

1.3. THEORETICAL FRAMEWORK

In this study we propose to use patient-derived iPSCs as a replacement source for primary human cardiomyocytes and an *in vitro* model for cardiac toxicology investigations.

Traditionally, it has been difficult to obtain primary human cardiac cells for research due to the rarity of healthy donor material. Culturing such cells is also difficult due to issues associated with the non-proliferative state of terminally differentiated cardiomyocytes. The identification and development of stem cell technology in the last 20 years however has enabled major advancements to be made in offering scientists a replenishable source of human material to study both healthy and diseased states.

Stem cells are unspecialised cells within the body that have the ability to develop into specialised cell types. Human pluripotent stem cells, including embryonic stem cells (ESCs) and iPSCs, have the potential to theoretically become almost any cell type and can maintain this ability whilst being cultured *in-vitro* indefinitely [51-53].

The first human ESC line was developed in 1998 from cultured human blastocysts, almost twenty years after the first murine ESC line was produced [52]. The derivation and use of ESCs in research however has posed a long-term ethical dilemma which has limited their use and the development of their application for clinical-based therapies. Scientists had found a way to produce a long-desired replenishable source of material, but its use was restricted by both moral and legal obligation.

In a more recent breakthrough, this problem was overcome in 2006 when Shinya Yamanaka and colleagues were able to produce mouse iPSCs from mouse skin fibroblasts by retroviral transduction of four transcription factor genes found to be upregulated in ESCs [51]. A year later they generated the first human iPSCs in the same way [54]. Indirectly influenced by the earlier advancement of ESC development the group theorised that the factors contained within ESCs, which can confer totipotency or pluripotency to somatic cells and which play important roles in ESC identity, would also

play pivotal roles in the induction of pluripotency in somatic cells. The group utilised knowledge obtained from published literature and prior studies to identify genes which are specifically expressed in ESCs as well as those which also contribute to the long-term maintenance and proliferation of ESCs in culture. Starting with twenty-four candidate genes, four essential factors were eventually identified which, when combined *in-vitro*, were able to generate pluripotent cells from a terminally differentiated cell type. The four factors (OCT3/4, KLF4, C-MYC and SOX2) became known in the field as the Yamanka factors. Simultaneous to the Japanese group making their breakthrough discovery, the American scientist who had influenced their work through his creation of the first human ESC line in 1981 was the first person to identify a different group of factors (OCT4, SOX2, NANOG, LIN28) to do the same [53]. Owing to their discovery, iPSCs today have many possible applications, including drug and toxicity screening, disease modelling, cell transplantation therapies, and regenerative medicine.

Prior to reprogramming, a suitable cell type must be chosen which is easy to obtain and susceptible to reprogramming. The first reprogramming using the Yamanaka factors was performed with fibroblasts but since its advent iPSC technology has undergone further advancement and more accessible cells than those requiring a biopsy, such as keratinocytes, peripheral blood cells, and renal epithelial cells from urine samples have been used for reprogramming [55].

As well as taking into consideration the invasive nature of obtaining a cell type of choice for reprogramming, the type of the somatic cell used also requires careful consideration as this choice can affect the transcription factors needed for successful reprogramming. For example, cells with high endogenous expression of SOX2 can be reprogrammed without SOX2, or even with OCT4 alone [56-58]. Using lesser amounts of reprogramming factors however also has an effect on reprogramming efficiency [59].

It has been shown that following reprogramming, iPSCs can still provoke an 'epigenetic memory' of the original donor cell [60-62]. As a result, upon initiating differentiation, iPSCs have a tendency to differentiate more easily into cells of the same germ layer as the original donor cell [60, 61]. Choosing a cell type for reprogramming from the same germ layer to that of the intended downstream target cell type can therefore help to improve differentiation efficiency [63].

The ability to generate iPSCs from patient samples makes it possible to study crucial aspects of a disease of interest and the technology has revolutionised the study of human cardiovascular science - the ability to attain unlimited numbers of cells in an ethically approved manner from both healthy donors and those with cardiovascular disease means that it is now possible to study cardiomyocytes *in-vitro* from a genetically relevant source.

There are many existing factors (or combinations of factors) that can be used for reprogramming. Many of the factors used to induce cellular reprogramming are factors that are normally expressed in the early embryo and play a role in maintaining pluripotency [64]. The original reprogramming cocktail used by Yamanaka and colleagues in 2006 consisted of four transcription factors all found to be upregulated in ESCs. The efficiency of reprogramming achieved was 0.02% with adult human dermal fibroblasts [51]. The Yamanaka factors are the most common factors used for reprogramming [65], however alternative combinations of reprogramming factors have also achieved successful reprogramming with varying levels of efficiency [66-68].

Once the cell type and reprogramming factors have been decided, a suitable reprogramming method also needs to be selected. Downstream application of the cells often determines the method of choice

for reprogramming as some of the most efficient methods rely on viral integration into the host genome, which would be unsuitable for clinical application. The reprogramming methods therefore often fall into two major classes - integrating and nonintegrating - depending on whether the reprogramming factors are incorporated into the host cell genome during reprogramming [64]. Higher quality iPSCs are produced by non-integrating methods as there is no risk of insertional mutagenesis or reactivation of the pluripotency genes.

The first successful reprogramming reported utilised a retroviral transduction method using Moloney murine leukaemia virus (MMLV)-derived retroviruses such as pMXs, pLib12 or pMSCV [51]. These viruses can infect dividing cells at an efficiency of 90% [64]. Nonetheless, reprogramming efficiencies using the Yamanaka factors reported for human cells is between 0.01-0.02% [69].

Another retroviral method used for reprogramming is transfection with lentiviruses derived from the human immunodeficiency virus (HIV). Lentiviruses have a higher infection efficiency and cloning capacity than the MMLV retroviruses and have become a more preferred method for generating iPSCs over the MMLV-retroviral method as it can infect both non-dividing and dividing cells [70]. The higher efficiency has been reported to be between 0.1-2% [64].

The major downside to using retroviruses is that the viral transgenes have been reported to integrate randomly into the iPSC genome, which could cause dysregulation of proto-oncogenes and insertional mutagenesis in the host cell genome [64]. The risk of insertional mutagenesis increases with the use of multiple transcription factors. An additional disadvantage with retroviruses is that the viral transgenes need to be silenced after iPSC formation for full reprogramming to be achieved [71], which can often be difficult to do. It is also possible that some viral transgenes may not be fully silenced at all leading to the potential of reactivation in the host genome at a later point [63, 72].

Due to the safety issues associated with the use of retroviruses, other methods of generating 'footprint-free' iPSCs have been developed. Non-integrating viral methods include transfection with Adenoviral or Sendai-viral vectors. The reprogramming efficiencies using replication deficient Adenoviruses have been as low as 0.0002% with human cells [73] and would require further optimisation to have useful application in iPSC generation [70]. A second option, an F-gene deficient form of the single-stranded negative-sense RNA Sendai-virus, has been shown to infect a wide range of host cells [74] and produce protein in large quantities [70]. The virus replicates in the host cell cytoplasm making it a more appealing, integration-free candidate for reprogramming. Moreover, the viral RNA is typically lost from the host cell by approximately passage 10, creating footprint-free iPSCs [70]. The viral particles can be removed by antibody-mediated negative selection against surface protein HN on the virus [75]. Human fibroblasts and blood cells have been reprogrammed using Sendai-virus with efficiencies of 0.1% and 1% [75-77] comparable to the lentiviral method but producing iPSCs of higher quality.

The first challenge of early iPSC research was to define whether the cells truly resembled ESCs. This was proven to be true morphologically, functionally, transcriptionally, and epigenetically [78-82]. The epigenetic differences observed in some iPSC lines compared to ESC lines were shown to be caused mainly by the reprogramming method used [83], and can be diminished during passaging of the iPSCs [84-86].

To assess the quality of the generated iPSCs, they must be characterised on many different levels. The first sign of iPSC formation is the typical morphology defined by compact colonies with defined

borders, having small cells with a high nucleus to cytoplasm ratio and large nucleoli [52]. For feeder-free monolayer cultures, the morphology is less defined [63]. In addition to the typical morphology, iPSCs are also known to proliferate extensively in culture [52].

In addition to morphological characterisation, many cellular and molecular assays are used to characterise the cells [63]. iPSCs are only considered to be fully reprogrammed when the transgenes are silenced and the endogenic pluripotency genes are turned on [71]. The silencing of the transgenes therefore needs to be confirmed and the expression of various pluripotency markers need to be assessed at the mRNA and protein level. The presence of one marker is not necessarily an indication of complete reprogramming [87], and many markers are often used. While many different assays can be used to characterise the created iPSCs, no method alone is sufficient to confirm good quality of the iPSCs. Thus, a combination of methods should be used [63].

The ability to generate iPSCs from patient samples makes it possible to study crucial aspects of a disease of interest and the technology has revolutionised the study of human cardiovascular science - the ability to attain unlimited numbers of cells in an ethically approved manner from both healthy donors and those with cardiovascular disease means that it is now possible to study cardiomyocytes *in- vitro* from a genetically relevant source.

In 2020 already, over 2500 manuscripts have been published which include the relevant search term "induced pluripotent stem cells cardiomyocyte". In 2019, over 10,000 manuscripts were published on the topic. The use of iPSCs to study cardiomyocytes is a rapidly expanding field with increasingly evolving potential – a theoretical framework that presents a robust method for studying the characteristics of human cardiomyocytes *in-vitro* from both healthy and diseased backgrounds.

2 STUDY OBJECTIVES

The overarching questions of our research are:

1) What are the mechanistic differences between patients who develop doxorubicin-induced cardiotoxicity and those who do not?
2) How do these differences drive cardiotoxicity?
3) Why do they drive cardiotoxicity in some patients and not others?
4) Can we predict which patients will develop cardiotoxicity before they become symptomatic?

In this pilot study we have identified three key aims, detailed below, that will allow us to generate preliminary data for the purpose of developing large-scale studies which will be focused towards answering these questions.

2.1 AIMS AND OBJECTIVES

2.1.1 Aim 1

To generate cardiomyocytes from patient-derived iPSCs.

Objectives

To achieve this, we will:

- Separate peripheral blood mononuclear cells (PBMCs) from patient blood samples.
- Reprogram PBMCs into iPSCs cells using the four Yamanaka transcription factors which will be introduced via Sendai-virus transduction or electroporation.
- Characterise and validate iPSC lines using microscopy, immunocytochemistry, and polymerase chain reaction (PCR).
- Develop reprogrammed iPSCs into patient-specific cardiomyocytes.
- Characterise and validate cardiomyocyte lines using microscopy, immunocytochemistry, PCR, and electrophysiology.

2.1.2 Aim 2

To characterise the mechanistic differences between patients with and without anthracycline-induced cardiotoxicity.

Objectives:

To achieve this, we will:

- Conduct proteomic profiling studies in cardiomyocytes generated from test subjects to detect short and long-term changes in protein expression levels.
- Characterise the electrical activity and calcium homeostasis of cardiomyocytes.
- Measure ionic currents, along with cardiac action potential to investigate morphological and pathophysiological changes in whole cell electrical signalling.
- Measure intracellular Ca^{2+} changes, intracellular ATP, mitochondrial function, and membrane potential.
- Analyse differences in mechanical beating behaviour between cardiomyocytes.

2.1.3 Aim 3

To Identify and characterise novel candidate biomarkers.

Objectives

To achieve this, we will:

- Use iPSCs to characterise biomarkers that can be readily validated within cells and secreted into media.
- Compare these to biomarkers found in blood obtained from patients.
- Compare the findings to the current method of detection of biomarkers at the participating hospital site (LHCH) to potentially identify new biomarkers through blood-based proteomics.

3 STUDY DESIGN

3.1 TYPE OF STUDY

This is a physiological case control study which aims to investigate the functional differences between iPSC-derived cardiomyocytes from two study groups.

3.2 DURATION

Funding duration for this study: 2 years

Recruitment duration for this study: 1 year

3.3 OUTCOME

Our primary outcome for this study is to generate iPSC-derived cardiomyocytes that are reproducible, amenable to experimental analysis, and that recapitulate the phenotype of the donor at the molecular and cellular level. This will not only allow us to begin addressing the research questions set out in this study of being able to better understand chemotherapy-induced cardiotoxicity, but will also enable us to use the technology to investigate other cardiac conditions and congenital defects.

Secondary outcomes include:

- Identifying a doxorubicin-specific signature in the cells derived from our cardiotoxicity subjects which signifies a distinct biological process that is absent in the control group. This would allow us a potentially significant insight into the mechanistic effects of doxorubicin-induced cardiotoxicity at a cellular and molecular level.
- Identifying candidate biomarkers that are of a functional relevance to doxorubicin-induced cardiotoxicity. Novel biomarker discovery could potentially offer an enhanced alternative to the current standard of risk stratification and cardiotoxicity detection in the clinic.
- Obtaining data that will support further investigations into different areas of future research - such as the role of candidate biomarkers that could predict anthracycline-induced cardiotoxicity in other cancers and what their relationships are to cumulative drug dosing strategies for example.

These outcomes could eventually enable clinicians to better predict which patients will develop cardiotoxicity and how their care can be more appropriately managed. The research will deepen the understanding of doxorubicin-induced cardiotoxicity for health care professionals and service providers for integrated care. It will also assist in providing a better understanding of the options available for supporting chemotherapy patients at various stages of their care both during and following the completion of their treatment. It has the potential to improve the wellbeing and lives of patients as well as to improve how primary and secondary care services work together. The study will develop high quality data that will be disseminated with researchers, service users and health care professionals to create more effective practices in care.

3.4 STUDY SETTING

In this multicentre study we will recruit participants from three NHS outpatient services (LHCH, CCC, RLBUHT) with the help of clinicians and staff members working across these sites. Participants will be actively identified and recruited at all sites as follows:

- Liverpool Heart and Chest Hospital will identify and recruit doxorubicin-treated chemotherapy patients who do or do not exhibit cardiotoxicity as determined by the appropriate clinical co-investigator (named on page vii of this document).
- Clatterbridge Cancer Centre and the Royal Liverpool and Broadgreen University Hospitals NHS Trust will refer doxorubicin-treated chemotherapy patients to LHCH for further examination.

LHCH provides specialist services in cardiothoracic surgery, cardiology, respiratory medicine and diagnostic imaging across Merseyside, Cheshire, North Wales, and the Isle of Man. Its outpatient department sees an estimated 70,000 patients a year and conducts a recently introduced consultant-led cardio-oncology clinic which see approximately 30 new referrals per month.

The Clatterbridge Cancer Centre NHS Foundation Trust is one of the UK's leading cancer centres and provides specialist cancer care across Cheshire, Merseyside and the surrounding areas including the Isle of Man. The centre provides non-surgical cancer care for blood cancers and solid tumours and operates specialist chemotherapy clinics in local district hospitals, including one of our participating sites - RLBUHT. The new Clatterbridge Cancer Centre, situated in central Liverpool alongside the University of Liverpool and Royal Liverpool University Hospital, will more greatly support recruitment opportunities of non-cardiotoxicity patients should it be necessary, and increase sample processing efficiency at the neighbouring University site.

The RLBUHT oncology services are world renowned and offer treatments for most types of cancer. The hospital works in collaboration with Clatterbridge and LHCH to deliver up-to-date care for cancer patients.

Each hospital site has the support and involvement of highly experienced expert consultants in both cardiology and oncology. The participating clinicians at LHCH (JW, RD) are both consultant cardiologists with specialist clinical and academic interests in cardio oncology, whilst the participating clinician at both CCC and RLBUHT (CP) is a specialist consultant in medical oncology.

The University of Liverpool conducts world-leading personalised health research across all fields of translational science and the staff members on this study have extensive academic and research experience in pharmacology, oncology and cardiovascular science, as well as experience in patient sample processing for downstream techniques. The University is also part of a strategic research collaboration and, along with Liverpool John Moore's University, LHCH and Liverpool Health Partners, forms part of the LCCS. LCCS brings together the region's experts with the aim of advancing cardiovascular research. The director of the LCCS (GL) is a consultant cardiologist at LHCH and a co-applicant of this study.

The location, staff expertise and specialty services of these sites makes each setting excellently suited to assist in addressing our research aims.

3.5 RECRUITMENT

3.5.1 Participant identification

Potential participants will be identified by the clinical co-investigators, as named on page 8 of this document, at LHCH. Identification of potential participants will be based on ejection fraction measurements as determined by the results of a retrospective 3D echocardiogram. Patients who have been referred to LHCH cardio-oncology clinic with suspected doxorubicin-induced cardiotoxicity will have received a 3D echocardiogram and blood-based biomarker test at the time of their outpatient appointment and either been diagnosed with cardiotoxicity or discharged back to their referring oncologist with negative test results. LHCH will approach those who fit the inclusion criteria from this selection of patients to be involved in the study. Once eligible cardiotoxicity participants have been identified at LHCH, non-cardiotoxicity subjects will be identified based on their retrospective test

results and matched to the cardiotoxicity subjects on tumour type, age, and treatment regime (including dosage and time frame). Non-cardiotoxicity subjects will be approached to take part as a control cohort by one of the named clinical co-investigators.

Eligible participants will be given basic information about the study by the co-investigator either during their outpatient appointment, over the phone, or by an invitation letter sent in the post. Patients who are interested in finding out more about the study will be given a Patient Information Sheet and the option to leave their telephone number for the clinical team to make contact, or to contact the clinical team themselves using the contact information given on the PIS.

Participants will not be recruited through Patient Identification Centres, disease registers, or through media advertising such as posters, leaflets, adverts, or websites. Participants will not receive any payment for participation in this study.

Due to the variation in reprogramming efficacy of stem cells, we may approach patients for a separate blood sample at a second clinical encounter. If patients do not want us to do this, they have the option to opt out at the time of consent and we will make a record of this in the site master file alongside their consent form; they will not be re-contacted. If, however, they are willing to provide a second sample, where possible we will take this during a routine outpatient appointment, however if this is not possible, we will arrange a research clinic appointment at LHCH.

A minority of patients may have already been consented prior to amending the protocol to allow us to re-approach patients for a second blood sample. In this instance we will contact the patient once via telephone and enquire whether they would be willing to give a second sample. If they do not want to give a second sample, we will make a note of this in the site master file and they will not be re-contacted. If they are willing to provide a second sample, where possible we will take this during a routine outpatient appointment, however if this is not possible, we will arrange a research clinic appointment at LHCH. We will use an additional study consent form for the second sample and for the question '3. If required, I agree to be re-contacted once for further blood samples' we will ask them to write 'n/a'. This second consent form will be stored alongside their first consent form in the site master file.

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3.6 SAMPLING

3.6.1 Size of sample

The maximum sample size for this pilot study at this stage will be 12 participants in total across all experimental groups (Figure 1). When selecting a sample size for this study we considered several factors including the nature of the study (i.e. a pilot study) and the financial feasibility of achieving our research aims at this stage. As this is a pilot study, we want to generate preliminary data that can support further funding applications that build on scientific evidence and eventually allow for larger sample sizes whilst still working realistically within the constraints of the study. Our approach is therefore to focus our efforts on obtaining a carefully planned smaller sample size which adequately represents significant aspects of the wider study population. This sample size is large enough to sufficiently address the primary research aims at the current stage and will provide valuable preliminary data to plan larger subsequent studies which will further address the overarching research questions.

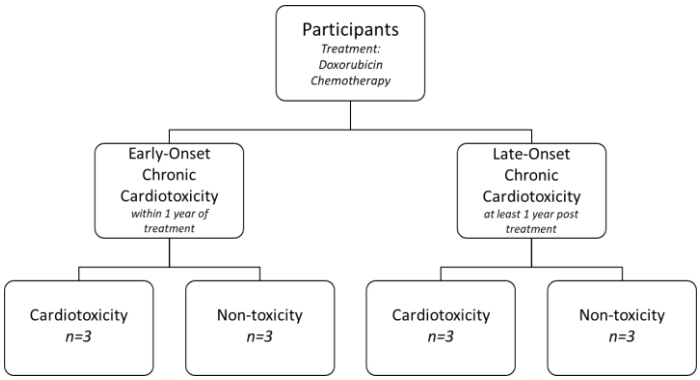

Figure 1. *Cancer patients with early-onset chronic cardiotoxicity and late-onset chronic cardiotoxicity will be recruited. Non-cardiotoxic patients will be matched to the cardiotoxic patients on tumour type, treatment regime and approximate age. For late-onset chronic cardiotoxic patients, non-cardiotoxic patients will be recruited who have previously received the same treatment regime and not developed cardiotoxicity at the same point.*

3.6.2 Sampling technique

The selection of participants will be purposive from a population of outpatients across three NHS hospital trust sites. This method of sample selection has been chosen to allow us to obtain information from a limited number of individuals within specific groups of interest (i.e. doxorubicin-treated patients who do and do not exhibit cardiotoxicity). Representative samples of interest will be selected by an experienced authority at each participating site. Whilst non-randomised sampling such as this may allow for selection bias, it also limits the possibility of sampling error, and data generated from the study will enable us to conduct larger studies where a better representation of the entire study population will be achievable through access to larger sampling numbers.

3.6.3 Sample collection

A venous blood sample will be taken from the antecubital area of the arm by a health care assistant or research nurse with experience of the venepuncture procedure. Samples will be collected in blood tubes which are appropriate for the end-point assay and used to isolate peripheral blood mononuclear cells and plasma. Once the sample has been drawn, to best preserve cell integrity, it will be maintained at room temperature (15-25°C) and processed within 24 hours of collection. Where possible, research samples will be taken with clinical samples during outpatient appointments to minimise the inconvenience to the participant. Where this is not possible, a research clinic will be arranged at LHCH to sample participants that have no pre-scheduled outpatient appointments.

3.6.4 Sample transfer and processing

Following sampling at LHCH, all blood tubes will primarily be stored under the relevant conditions in the research laboratory at LHCH before being collected on the same day by the study's designated research assistant and transferred to the University of Liverpool's main campus for processing. If a participant is sampled at one of the study's other participating hospital sites (CCC and RLBUH), which are situated adjacently to the University of Liverpool's main campus, then those samples will be collected immediately by the study's designated research assistant and transferred across to the University of Liverpool for processing. All samples transferred to the University of Liverpool will be documented on a transfer log and their end-location will be recorded. All samples will be processed by the study's research assistant at the University of Liverpool in the Sherrington Building's primary tissue culture suite.

3.6.5 Sample storage and tracking

After processing, surplus peripheral blood mononuclear cells from all participants will be frozen for long-term storage at -150°C under HTA guidelines. Plasma and nucleic acid samples will also be stored from all participant samples and will not be considered 'relevant material' under the HTA Act 2004. All samples (cells, plasma, nucleic acids) will be stored at the Clatterbridge Cancer Centre-Liverpool under the custodianship of the project's CI. Sample processing will be logged on a secure Microsoft Excel database and the details will be replicated in a linked logbook which will be kept in a secure drawer in a locked office at the University of Liverpool. The CI and research assistant will have access to the samples and access to the secure database which will allow for monitoring of their location during storage and subsequent use. No whole blood will be stored. Samples may be stored for future unspecified research with ethical approval.

4 PARTICIPANT ENTRY

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4.1 PRE-REGISTRATION EVALUATIONS

Potential participants for this study will have received clinical evaluations as part of their oncology/cardiology treatment, such as base-line blood tests at one or more participating hospital site, a 2D echocardiogram at one or more participating hospital site, and a 3D echocardiograms at LHCH.

4.2 INCLUSION CRITERIA

4.2.1 Cardiotoxicity Cohort

- Over 16 years of age at the time of consent.
- Capable of providing informed consent as determined by the consenting clinician.
- Receiving doxorubicin chemotherapy treatment at the time of consent or previously received doxorubicin chemotherapy treatment prior to consent.
- Clinical presentation of left ventricular systolic dysfunction at the time of consent secondary to receiving doxorubicin chemotherapy treatment as determined by the clinical team.

4.2.2 Non-cardiotoxicity Cohort

- Over 16 years of age at the time of consent.
- Capable of providing informed consent as determined by the consenting clinician.
- Receiving doxorubicin chemotherapy treatment at the time of consent or previously received doxorubicin chemotherapy treatment prior to consent.
- Normal left ventricular systolic function at the time of consent secondary to receiving doxorubicin chemotherapy treatment as determined by the clinical team.

4.3 EXCLUSION CRITERIA

- Under 16 years of age at the time of consent.
- Lacking ability to provide informed consent as determined by the consenting clinician.
- Judged to have been coerced to consent as determined by the consenting clinician.
- Pre-existing left ventricular systolic dysfunction to be reviewed by the clinical team on a case-by-case basis.
- Recent surgery (<3 months).
- Excessive alcohol consumption (>30 units a week) and/or recreational drug use.
- Active immunological disease as determined by the clinical team.
- On current steroid therapy with the exception of corticosteroid inhaler <2mg/kg.
- Active or recent (<6 weeks) serious infection as determined by the consenting clinician.
- Inability to comply with study procedures.

4.4 WITHDRAWAL CRITERIA

Participants may be withdrawn from the study if blood samples are unattainable or are unsuitable for laboratory analysis. Participants may withdraw consent to take part in the study at any time and their routine clinical care will continue unaffected and without prejudice. If a participant who has given informed consent loses capacity to consent during the study, the participant would be withdrawn from the study. Identifiable data or samples already collected with consent would continue to be retained and used in the study and participants will be made aware of this at the time of consent. Nofurther data or samples will be collected, or any other research procedures carried out on or in relationto the participant following withdrawal from the study.

~~5~~4 ADVERSE EVENTS

5.1 DEFINITIONS

Adverse Event (AE): any untoward medical occurrence in a patient or clinical study subject

Serious Adverse Event (SAE): any untoward and unexpected medical occurrence or effect that:

- **Results in death**
- **Is life-threatening**
- **Requires hospitalisation, or prolongation of existing inpatients' hospitalisation**
- **Results in persistent or significant disability or incapacity**
- **Is a congenital anomaly or birth defect**

Medical judgement should be exercised in deciding whether an AE is serious in other situations. Important AEs that are not immediately life-threatening or do not result in death or hospitalisation but may jeopardise the subject or may require intervention to prevent one of the other outcomes listed in the definition above, should also be considered serious.

5.2 REPORTING PROCEDURES

All adverse events should be reported. Depending on the nature of the event the reporting procedures below should be followed. Any questions concerning adverse event reporting should be directed to the CI in the first instance.

5.2.1 Non serious AEs

All such events, whether expected or not, should be recorded

5.2.2 Serious AEs

An SAE form should be completed and sent to the CI within 24 hours. However, relapse and death due to cancer or CVD, and hospitalisations for elective treatment of a pre-existing condition do not need reporting as SAEs.

All SAEs should be reported to the relevant REC where in the opinion of the CI, the event was:

- 'related', i.e. resulted from the administration of any of the research procedures; and
- 'unexpected', i.e. an event that is not listed in the protocol as an expected occurrence

Reports of related and unexpected SAEs should be submitted within 15 days of the CI becoming aware of the event, using the NRES SAE form for non-IMP studies. The CI must also notify the Sponsor of all SAEs.

Local investigators should report any SAEs as required by their Local Research Ethics Committee, Sponsor and/or Research & Development Office.

Contact details for reporting SAEs:

Please send SAE forms to Dr Parveen Sharma

Tel: 0151 795 0149 (Mon to Fri 09.00-17.00)

Email: Parveen.Sharma@liverpool.ac.uk

~~6~~5 ASSESSMENT AND FOLLOW-UP

6.1 FOLLOW UP

There will be no participant follow up for this study in a research capacity. Participants will continue to receive any routine clinical follow-up independently and unrelated to this study.

6.2 DATA ANALYSIS

6.2.1 Clinical data analysis

Patient demographics and clinical test results will be recorded by the clinical team for the purposes of the study and will be accessible to the clinical team only. A list of the relevant clinical data that will be collected can be found in Appendix 13.1.3. Access to patient records to identify potential participants and check whether they meet the relevant inclusion criteria will be restricted to the patient's existing clinical care team only - which in all cases will be one of the three named co-investigators on page 8 of this document or, on occasion and at their discretion, their appointed research nurse.

6.2.2 Experimental data analysis

All experimental methods carried out during this study will be conducted at the University of Liverpool by the study's designated research assistant. All experimental data generated will be processed and analysed using University equipment and any appropriate commercially available software as deemed necessary by the study's PI. All experimental data generated will be stored on computers and hardware owned by the University of Liverpool and will be directly accessible to the study's PI and research assistant for analysis and interpretation. No personal identifiers belonging to any participant will be kept at the University of Liverpool.

6.2.2.1 Cell lines

All cell lines used in this study will be generated from somatic patient cells. The cells used for reprogramming will be PBMCs isolated and cultured from whole blood. Cells will be reprogrammed using either Sendai-virus transduction or by electroporation of episomal plasmid vectors. Cell lines

generated will be allocated a pseudoanonymised form of identification by the research staff at the University of Liverpool. Surplus primary material (PBMC) will be cryopreserved in cryopreservation medium. Cells will be frozen in isopropanol containers at -80°C for 24 hours before being transferred to long-term storage at -150°C under HTA guidelines. All samples will be stored at the University of Liverpool.

6.2.2.2 Cellular Reprogramming

Sendai lines will be transduced with the CytoTune iPSC Sendai Reprogramming Kit (Invitrogen), which contains F-gene deficient Sendai-virus expressing the four Yamanaka transcription factors (SOX2 OCT3/4 c-MYC and KLF4). The reprogramming will be conducted according to manufacturer's instructions. After transduction, the Sendai-lines will be re-plated onto cell-free matrix-coated 6-well plates and cultured until iPSC colonies are ready to differentiate.

Electroporation lines will be transfected with integration free, episomal plasmid vectors containing pluripotency genes l-MYC, OCT3/4, SOX2 and KLF4 using a Nucleofector system (Lonza) according to the manufacturer's instructions. Transfected cells will be cultured under the appropriate culture conditions and then re-plated onto cell-free matrix-coated 6-well plates and cultured until iPSC colonies are ready to differentiate.

Throughout the reprogramming and cell maintenance period, we will cryopreserve stocks of iPSC lines in cryopreservation medium. Cell lines will be frozen in isopropanol containers at -80°C for 24 hours before being transferred for storage at -150°C at the University of Liverpool.

6.2.2.3 Microscopy

Brightfield microscopy will be used to analyse cellular reprogramming and differentiation efficiency as well as to assess the regular progress and overall health of the cells whilst in culture. Reprogramming efficiency will be assessed by microscopy in two ways – firstly, following transduction/transfection, all newly generated iPSC colonies will be counted and divided by the number of input cells used for reprogramming. Secondly, the number of iPSC colonies that survive following picking will be divided by the number of colonies that are selected for picking. The cardiomyocyte differentiation efficiency will be determined by dividing the number of observed beating areas by the total number of iPSC colonies that are used for differentiation.

Overall cell health will be assessed through regularly examining the morphology of the cells. Reprogrammed iPSC colonies will be examined for typical features indicative of good health such as dense, flat, rounded colony formation with even colouring and a high nucleus-to-cytoplasm ratio.

Microscope data on cell morphology, reprogramming efficiency and differentiation efficiency will be collected using and Invitrogen EVOS XL Core imaging system and analysed using an appropriate imaging software programme.

6.2.2.4 Polymerase Chain Reaction (PCR)

We will use PCR to characterise the gene expression of iPSC and cardiomyocyte cell lines. PCR data will be generated using several established techniques. Standard PCR and RT-PCR will be used to confirm the absence of exogenic genetic material from electroporated and Sendai-virus iPSC lines, respectively. The expression of EBNA-1, which will be present in the transfection plasmids, will be

assessed for electroporated lines. For Sendai-virus lines, the four viral transgenes (KLF-4, SOX-2, c-MYC and OCT-3/4) will be assessed. After verifying the absence of exogenic genetic material, the expression of endogenic pluripotency genes will be studied at the mRNA level by RT-PCR. GAPDH will be used as an endogenic control. Nucleic acid samples will be collected using commercially available reagents and collected samples will be stored at -80°C until extraction. Nucleic acid extraction will be performed using a commercially available extraction kit according to the manufacturer's instructions. Sample concentration will be measured spectrophotometrically and extracted samples will be stored at -80°C. RNA samples will be transcribed into cDNA using a commercially available kit according to manufacturer's instructions and synthesised cDNA will be stored at -80°C until ready for use. PCR products will be separated and analysed using agarose gel electrophoresis and the results viewed using a UV gel documentation system. The data images will be further interpreted using an appropriate imaging software programme.

To obtain quantitative PCR data which will allow us to compare the expression of pluripotency genes both within the same subject and between individual subjects, we will use a fluorescent reporter probe-based qPCR method. cDNA samples will be synthesised as described above from cell lines at two different passages (early and late) and studied to detect the expression levels of endogenous pluripotency genes with GAPDH levels used as an endogenic control. Samples will be processed in triplicate and analysed using the double-delta ct method to calculate relative expression. Statistical analyses will be performed to study (1) the differences in relative gene expression at early and late passages in the same subject (Mann-Whitney U test) and, (2) significant differences between subjects at the same passage number (Kruskal-Wallis test). Statistical analysis will be conducted using the R computational software environment.

6.2.2.5 Karyotyping

To determine the chromosome complement of the cells we will carry out chromosomal karyotyping using a commercial protocol or an outsourced commercial karyotyping service.

6.2.2.6 Immunocytochemistry

We will use indirect immunocytochemistry (ICC) to characterise iPSC and cardiomyocyte cell lines at the protein level. This method will allow us to generate data that will confirm the simultaneous expression and cellular location of key pluripotent stem cell markers and cardiac-specific markers such as Troponin-T. The stained cells will be viewed using a fluorescence microscope and the images will be captured using a digital camera. Captured images will be interpreted and analysed with the aid of an appropriate imaging software, such as Adobe Photoshop, and data may be manipulated to include the addition of scale bars, contrast adjustment and image overlays. Data manipulation will not affect the result of the experiments in any way. Our interpretations of the data will be validated with the use of positive and negative staining controls and imaging parameters for the controls will be the same as those applied to the test dataset. In addition to obtaining data on protein expression levels, the cardiomyocyte differentiation efficiency will also be assessed by ICC. A select number of images (<5) will be captured at random and the number of cardiac TroponinT- positive cells will be divided by the total cell count. Cells will be counterstained with a nucleic acid stain (DAPI) to achieve this.

6.2.2.7 Mass Spectrometry (MS)

To generate quantitative data on the whole proteome of doxorubicin-treated cardiomyocytes, cardiomyocytes from all experimental groups will be subjected to iTRAQ-based MS. To determine whether protein expression is similar between cardiotoxicity and non-cardiotoxicity samples, the data will be subjected to principal component analysis to analyse the variance across the sample sets and identify whether there is a distinction between them based on protein expression. A two-tailed *t* test will be carried out using the R computational environment to determine the statistical significance of differences in protein expression between cardiotoxicity and non-cardiotoxicity samples

To validate the iTRAQ-MS data, we will use Western blot analysis to verify the differential protein expression of a select number of identified proteins. The identity of the target proteins will be confirmed by comparison to a molecular weight marker (for size) and a positive control if possible (for size and signal). A loading control will be used to allow us to normalise the data and compare the expression levels between the target proteins. The data produced will be interpreted using imaging software, such as ImageJ, and a semi-quantitative comparison will be made of the signals generated between protein bands.

Following validation, proteins which are found to have a statistically significant higher or lower level of expression by MS in the control samples will be explored further using computational enrichment analysis to identify functional pathways involved in cardiotoxicity.

6.2.2.8 Electrophysiology

We will use standard electrophysiological and fluorescence imaging techniques to characterise the electrical activity and calcium homeostasis in iPSC and cardiomyocyte cell lines. Patch clamp recordings will be made from single cells using microelectrodes formed from thick walled borosilicate glass filled with an electrolyte solution connected to an industry standard amplifier (Axopatch 200B), digitised (Digidata 1440) and recorded using specialised electrophysiological recording software (pCLAMP10.7). It is anticipated that we will measure ionic currents, in particular K^+ and Ca^{2+} along with the cardiac action potential to investigate morphological and pathophysiological changes in the whole cell electrical signalling that may correlate with disease states. In particular, current amplitude will be measured using voltage-protocol appropriate to the current under investigation. Action potential amplitude and duration will be measured as these are common markers of electrical dysregulation.

In order to study the responses to electrical signalling, we will use state-of-the-art fluorescence measurements to measure intracellular Ca^{2+} changes correlating to each contractile cycle (using fluo-3, fluo-4 or Fura-2), along with intracellular ATP (using MgGreen), mitochondrial function (using TMRE/TMRM) and membrane potential (using Di-4-ANNEPS or Di-8-ANNPES) across the syncytium of cells using cell permeant fluorescent markers. Fluorescence will be excited using a PTI monochromator attached to a Nikon TiU microscope with fluorescence signals detected using a Andor Zyla camera controlled by Winfluor4.5 software. In all cases, daily control data sets will be gathered along with any test data sets. Calcium fluorescence signals from either spontaneous action potentials or from electric field stimulation for pacing of cells, will be analysed for peak amplitude, transient duration and measurement of the area under the curve to assess changes. ATP and mitochondrial membrane potential will be measured for responsiveness to simulated ischaemia, where cells exposed to toxic conditions generally show an increased rate of mitochondrial depolarisation and rapid ATP depletion. Finally, membrane potential indicators will be used to measure the spread of excitation through the syncytium. Measurements of the rate of depolarisation and the delay across the syncytium will be

assessed. These protocols are well established in the group, and standard data analysis protocols and statistical analysis are established and are used in peer-reviewed published manuscripts.

All raw electrophysiological recordings will be available in pCLAMP format (.abf files), whilst all imaging data will be stored as time stamped image files (.tif) or image stacks readable in ImageJ.

~~7~~6 ETHICAL & REGULATORY CONSIDERATIONS

7.1 ETHICS APPROVAL AND OTHER REGULATORY REVIEWS AND REPORTS

The CI will obtain approval from the appropriate Research Ethics Committee and Health Research Authority (HRA) approval for the study protocol, PIS, informed consent forms and other relevant documents with respect to the scientific content and compliance with the tenets of the Declaration of Helsinki prior to commencing recruitment. The study will be submitted to each proposed researchsite for Confirmation of Capacity and Capability. The study will be conducted in accordance with the recommendations for physicians involved in research on human subjects adopted by the 18th World Medical Assembly, Helsinki 1964 and later revisions

Substantial amendments that require review by NHS REC will not be implemented until that review is in place. All correspondence with the REC will be retained.

The CI will produce the annual reports as required and notify the REC of the end of the study. If an annual report is required for this study, an annual progress report will be submitted to the REC within 30 days of the anniversary date on which the favourable opinion was given, and annually until the study is declared ended. If the study is ended prematurely, the CI will notify the REC, including the reasons for the premature termination. Within one year after the end of the study, the CI will submit a final report with the results, including any publications/abstracts, to the REC.

7.1.1 Regulatory Review & Compliance

Before any participating site starts to enrol subjects into the study, the CI will ensure that appropriate approvals from participating organisations are in place.

For any amendment to the study the CI, in agreement with the Sponsor, will submit information to the appropriate body for them to issue approval for the amendment. The CI will work with participating sites to put the necessary arrangements in place to implement the amendment.

7.1.2 Amendments

Any substantial changes to the protocol which may impact on the conduct of the study, potential benefit of the patient or may affect patient safety, including changes of study objectives, study design, patient population, sample sizes, study procedures, or significant administrative aspects will require a formal amendment to the protocol. Such amendments will be agreed upon by the CI and the grant applicants and approved by the appropriate REC prior to implementation.

Administrative changes (minor corrections and/or clarifications that have no effect on the way the study is to be conducted) to the study protocol will be agreed upon by the CI and the grant applicants

and will be documented in a memorandum. The REC may be notified of administrative changes at the discretion of the CI.

7.2 CONSENT

To gain informed consent, a member of the clinical team will discuss with the potential participant, or his/her representative, the nature and objectives of the study as well as the potential risks and benefits associated with participating. Potential participants who are interested in finding out more about the study will be provided with a REC approved written (English) PIS whose content will be written in a way which is easy to understand and will include details relating to: the purpose of the study, the voluntary nature of participating, data collection and use of data, risks and benefits of participating, storage and reuse of samples, necessary contact information for both the clinical and research teams, withdrawal procedures, and how to file a complaint.

Potential participants will be given the opportunity to ask the clinical staff any questions and [if they wish] to discuss their interest in participating with others before choosing to take part. Capacity to consent will be assessed by the clinical team (i.e. the consenting clinician/research nurse). A member of the clinical team will assess a potential participant's capacity to consent through their understanding of the purpose of the research and its objectives, what the research involves, and what the potential risks and benefits of participating are. Potential participants will be asked whether they have discussed their decision to participate with anyone else to establish whether they have been coerced to participate. Individuals deemed to lack the capacity to consent or deemed to have been coerced to consent will not be enrolled on the study. Patients will be reassured that they can withdraw from the study at any time without the decision impacting on their clinical care.

Any samples collected prior to the point of withdrawal from the study will only be used with prior consent. All participants will be enrolled onto the study by signing a consent form. A hard copy of the consent form will be stored by both the participating hospital site at which the participant is enrolled and by the research team at the University of Liverpool. A copy will also be given to the participant. The consent form will include the following statements:

- That they have read and understood the information sheet
- Participation is voluntary and they can change their mind at any time
- That they understand that their care will not be affected by the decision to participate
- If any concerns about the participants ongoing health arise during the interview, we are obliged to disclose these to their GP

The protocol for re-approaching patients and consenting them is outlined in section 3.5.1. Patient recruitment.

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7.3 DATA PROTECTION AND PATIENT CONFIDENTIALITY

The CI will preserve the confidentiality of participants taking part in the study and will abide by the EU General Data Protection Regulation 2016 and Data Protection Act 2018. All co-investigators and studysite staff will comply with the requirements of the Data Protection Act 2018 with regards to the collection, storage, processing, and disclosure of personal information and will uphold the core principles of the Act.

Once they have entered the study and signed the consent form, participants will be assigned a unique study number by a research nurse. Their clinical and demographic details, as judged

relevant to the study by the clinical team and excluding any primary identifiers, will be entered into a secure database accessible to both the clinical team and the research team. All blood tubes used for sampling of a participant will be labelled with the participant's study number and the research team will be able to use this number to gain relevant information from the database for data analysis purposes, whilst also maintaining patient anonymity. The key that links a participant's study number to their primary identifiers (name, date of birth and NHS number) and the details of the key will be retained in secure facilities, separate from the data, and in accordance with data protection and information governance policies at the University of Liverpool and LHCH. Access to the key will be limited to the minimum number of individuals necessary for quality control and audit purposes.

All study-related information will be stored securely at the study site and all participant information will be stored in locked filing cabinets in areas with limited access and/or on password-protected computers. Sensitive personal data (e.g. sexual orientation, health records, religion, ethnicity, biometric data, personal identifiers etc) will be handled in such a way that no individual can be identified from the data without a coded ID which allows the data to be re-identified. This means that direct and indirect identifiers will be obscured or removed, and study records identified by the coded ID kept separately in a secure manner from study records containing any personal identifiers. Whilst it is acknowledged that pseudoanonymised data such as this is still considered personal data, this method is actively encouraged under GDPR security measures.

All clinical test results will be kept strictly confidential and clinical procedures will be conducted in a private room. Participants' study information will not be released outside of the study without the written permission of the participant.

7.4 INDEMNITY

The University of Liverpool holds Indemnity and insurance cover with Marsh UK LTD, which applied to this study.

7.4.1 Arrangements for insurance and/or indemnity to meet the potential legal liability of the Sponsor for harm to participants arising from the management of the research

The University of Liverpool holds professional indemnity and clinical trials insurance which applies to University-sponsored research. This covers the legal liability of the University as a research Sponsor in the eventuality of harm to a research participant arising from the management of the research by the University.

This does not affect a participating NHS Trust's responsibility for any clinical negligence on the part of its staff (including the Trust's responsibility for University of Liverpool employees acting in connection with their NHS honorary appointment).

7.4.2 Arrangements for insurance and/ or indemnity to meet the potential legal liability of the Sponsor or employer(s) for harm to participants arising from the design of the research

The University of Liverpool holds professional indemnity and clinical trials insurance which applies to University-sponsored research. This covers the legal liability of the University as a research Sponsor and/or employer of staff engaged in research, for harm to a research participant arising from the design of the research, where the research protocol was designed by the University.

Exceptions to the insurance policy that require prior approval from the Sponsor's insurers are:

- Recruitment of participants in the following groups:
  - Children under the age of 5
  - Pregnant Women
  - Participants who lack the capacity to consent
- First in Man (Phase I) CTIMPs
- Clinical Investigations of Medical Devices
- Studies including medical intervention involving contraception
- Clinically based Studies taking place at international sites
- Research being carried out at other organisations where the University is required to provide insurance cover
- Research being conducted by an external PI.

7.4.3 Arrangements for insurance and/ or indemnity to meet the potential legal liability of investigators/collaborators arising from harm to participants in the conduct of the research.

The University of Liverpool's insurance policies do not provide an indemnity to collaborators. As Research Sponsor, the University will ensure as far as reasonably practicable at the outset of the study that collaborators hold appropriate legal liability insurance.

The University of Liverpool's professional indemnity insurance policy provides an indemnity to its employees for their potential liability for harm to participants during the conduct of the research. Again, this does not in any way affect a participating NHS Trust's responsibility for any clinical negligence on the part of its staff (including the Trust's responsibility for University of Liverpool employees acting in connection with their NHS honorary appointment).

All NHS Trusts in England currently belong to the Clinical Negligence Scheme for Trusts (CNST) which covers the participating Trust for clinical negligence claims once proven in court.

All participating clinicians involved in the study hold appointments with either the Liverpool Heart and Chest Hospital, Clatterbridge Cancer Centre, or the Royal Liverpool and Broadgreen NHS Hospital Trust, giving them the protection of the NHS indemnity scheme.

7.4.4 Arrangements for payment of compensation in the event of harm to the research participants where no legal liability arises

No arrangements have been made for payment of compensation in the event of harm to the research participants where no legal liability rises.

7.5 SPONSOR

The University of Liverpool will act as Sponsor for this study. It is recognised that as an employee of the University the CI has been delegated specific duties, as detailed in the Sponsorship Approval letter.

7.6 FUNDING

The current pilot study is funded by the Liverpool Heart and Chest research fund.

7.7 AUDITS

The study may be subject to inspection and audit by the University of Liverpool under their remit as Sponsor and other regulatory bodies to ensure adherence to GCP and the UK Policy Framework for Health and Social Care Research (v3.2 10th October 2017).

7.8 ASSESSMENT AND MANAGEMENT OF RISK

The main risks associated with this study have been identified as follows:

- Patients enrolled without consent
- Failure to act on consent withdrawal request
- Failure to protect patient confidentiality
- Hazards of patient sampling methods

The key elements of the risk management plan in response are:

i. Measures to ensure informed consent is given prior to enrolment

A formal signed document will be used to obtain informed consent from all participants prior to enrolment. A clear record of what information has been conveyed, to whom, and when will be maintained in paper form by the co-investigator of the consenting site and the PI. All staff consenting will be experienced in consenting study participants and will take into consideration the subject's physical, emotional, and psychological capability when assessing and consenting a participant. Research staff will not process any samples without obtaining a hard copy of the signed consent form beforehand.

ii. Measures to ensure withdrawn consent is adhered to

The process of consenting is ongoing and will be made clear to the subject that it is their right to withdraw consent at any time, not just at the initial signing of paperwork. The procedure for withdrawing consent and the contact details of who to inform will be contained within the PIS. Clinical staff will inform the PI immediately of any participants who have withdrawn consent so that all stored material can be disposed of in accordance with HTA guidelines. A clear record of what information has been conveyed, to whom, and when will be maintained in paper form by the co-investigator of the consenting site and the PI. A record of all samples destroyed will be maintained by the PI and research assistant at the University of Liverpool on a secure, password-protected server. All research samples

will be pseudoanonymised at the time of processing and personal identifiers will not be recorded on the sample destruction log.

iii. Measures to ensure confidentiality of data

All information received by clinical and research staff will be treated as confidential unless there are safeguarding concerns. Hospital safeguarding policies will be followed by the relevant co-investigator if any concerns are raised. Responses on the patient consent form will be separated from any identifying personal data items, as already described, using a unique identifier for each response. Patient information will be transcribed and pseudoanonymised and stored securely on password-protected hospital servers. A key document linking names of participants to unique identifiers will be stored securely and separately from the pseudoanonymised data and destroyed once analysis has been completed. Only the study's co-investigators will have access to the key document.

Any data used in project reports or publications will be not be attributed to an identified individual without the written permission of that individual. Researchers will also ensure that the content of the material cannot be used to identify any individual.

Participant information sheets and consent forms will explain these measures to protect confidentiality

iv. Measures to ensure safe sampling

All staff tasked with sampling patients will be fully experienced and certified to do so.

7.9 PEER REVIEW

Peer review of this protocol was independently carried out by two individual experts with knowledge of the relevant discipline. The peer review process for this protocol was commensurate with the size and complexity of the study and met the standard outlined by The National Institute Health Research (NIHR) Clinical Research Network (CRN).

7.10 PATIENT AND PUBLIC INVOLVEMENT

As this is a pilot study, no aspects of the research process for this study have actively involved, or will involve, patients, service users, and/or their carers or members of the public.

7.11 PROTOCOL COMPLIANCE

Research team members are required to report any breaches of this protocol to the CI. Any reported incidents will be discussed at the next Study Management Team meeting where appropriate measures will be agreed to prevent recurrence. Any breaches reported will be documented along with the preventative measure suggested. New measures will be reviewed at subsequent meetings.

7.12 ACCESS TO THE FINAL DATASET

All data sets will be password protected. The CI will be given access to the full data set and will grant access to the full data set to any other participating investigator/member of staff as they judge

necessary for the purposes of the study. To ensure confidentiality, data dispersed to project team members will be blinded of any identifying participant information.

The CI and co-investigators at each participating site will have direct access to their own site's data sets and will have access to another site's data set by request to the CI.

It is envisaged that data sets from this study may be used for secondary analysis at a later point and its future use will be reflected in all patient documentation. Secondary analysis will only be undertaken with the informed consent of participants which will be acquired at the time of enrolment. Continued consent will be obtained prior to the point of secondary analysis. The purpose for using personal data for secondary analysis will also be communicated in the Privacy Notices.

~~8~~7 STUDY MANAGEMENT

The day-to-day management of the study will be coordinated through Dr Parveen Sharma

~~9~~8 END OF STUDY

The end of study will be defined as the completion of data collection and analysis.

~~10~~9 ARCHIVING

Data and all appropriate documentation will be stored for a minimum of 10 years after completion of the study, including the follow-up period, unless otherwise directed by the funder/sponsor/regulatory bodies. Data for this study will be stored on The University Active DataStore. This provides a centralised, secure, supported data storage facility for electronic data, with ongoing access for the life span of a project. Each new project application is sent for line manager approval before being forwarded to the RDM team.

~~11~~10 PUBLICATION POLICY

11.1 DISSEMINATION POLICY

All research data generated by the University of Liverpool will be wholly owned by the University (as the funder) and will remain with the University if the academic who generates the data leaves the institution. Where taught students generate data under the supervision of an academic member of staff as part of the project, the data will be wholly owned by the University as above. As it is not a condition of the grant or a contract, exclusive rights to the research data will not be assigned, licenced, or otherwise transferred to external parties.

On completion of the study, the data will be analysed and tabulated and an impact report will be submitted to the University of Liverpool TRAP Award Panel (funding panel) within three months of the date of completion of the project.

The determination of whether a data set represents a primary outcome will be made by the study's PI. All papers, abstracts and presentations containing data sets other than those designated as representing a primary outcome must be approved by the PI prior to submission.

In the event that participating investigators are asked to contribute data to workshops, symposia, etc - the individuals to work on such requests will be selected by the PI, but where time permits, a proposal will be circulated requesting the assistance of other individuals where necessary.

All presentations and publications are expected to protect the integrity of the major objective(s) of the study; Recommendations as to the timing of presenting endpoint data, and any meetings at which they might be presented, will be decided by the PI.

Every attempt will be made to reduce the interval between the completion of data collection and the release of the study results. Each paper and/or abstract will be submitted by the corresponding author to all listed authors and grant applicants for review of its appropriateness and scientific merit prior to submission. All listed authors will have authority on the content and may recommend changes to the corresponding author. All listed authors will also review the final version of the manuscript prior to submission. There are no publication restrictions to disclose.

The study results will be released to the participating researchers and clinicians, referring clinicians, and the general academic community via abstracts, manuscripts, and presentations at scientific meetings. The decision to notify participating subjects of the outcome will be at the discretion of the study's PI.

Study participants have the right to access their medical record and therefore the outcome of any clinical procedure conducted as part of the study can be requested at any time. A request for information pertaining to a clinical procedure carried out at any of the study's participating hospital sites can be accessed via the records manager or patient services manager at the relevant hospital trust. Study participants will be made aware of this contact information at the time of enrolment.

Study participants can request information from their participating site's co-investigator about the outcome of their own research data set, which can be explained to them by the co-investigator or the PI, but they will not be able to request a copy of any experimental data analysis. Individual experimental data results can be obtained by the co-investigators from the PI on request as soon as they become available.

11.2 AUTHORSHIP ELIGIBILITY GUIDELINES AND ANY INTENDED USE OF PROFESSIONAL WRITERS

Authorship criteria will be agreed by all grant applicants at an early stage of the research. Where possible, written records of decisions regarding authorship will be obtained and routinely revisited where roles and contributions change during the duration of the study. Topics suggested for presentation or publication will be circulated to the designated co-investigator at each participating study site as well as to the grant's co-applicants. This group will suggest and justify names for authorship. If a topic is suggested by a participating investigator, the person suggesting the topic may be considered as the lead author. Any individual who has made a substantive contribution to the inception or design of the project, or the acquisition, analysis, or interpretation of data will be recognised through authorship. Individuals who contribute to the work but who do not fulfil the

authorship criteria described above (e.g. nursing/auxiliary/technical staff etc) will not be granted authorship but will be properly acknowledged in the final manuscript at the suggestion of a study site's co-investigator. All acknowledgements will fully reflect the level of input of the contributor. Additionally, any guidelines on defined authorship criteria of the potential publishing journal will also be consulted if necessary. Disputes regarding authorship will be settled by the CI.

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~~13~~12 APPENDICES

13.1 Appendix 1- Required documentation

13.1.1 Patient Information Sheet (attached as a separate document)

13.1.2 Consent Form (attached as a separate document)

13.1.3 Clinical Data Collection List (attached as a separate document)

13.2 Appendix 2 – Amendment History

Amendment No.	Protocol version no.	Date issued	Author(s) of changes	Details of changes made
1	0.1	19.05.20	LS	First draft version sent to Study Management Team for comments
2	0.2	27.05.20	PS, JW, RD, GL, CP, RR	Contributor comments and changes returned for consideration
3	0.3	05.06.20	LS	Second draft version sent to Study Management Team for Peer Review distribution
4	0.4	18.07.20	LS	Addition of IRAS ID
5	1.0	27.07.20	LS	Final version for submission
6	2.0	13.09.20	LS	1. Sponsorship application committee request the use of the University Non-CTIMP protocol template instead of the HRA protocol template used for submission of v1.0. 2. Points raised by the Sponsorship application committee also addressed.

7	2.1	17.05.22	DG	1. Addition of D. Gent as co-investigator in the protocol text 2. Updating the protocol to change the source of the funding 3. Updating the protocol to include new contact details for the University of Liverpool sponsor.
8	2.2	15.09.22	DG	1. Amendment to text outlining how we would re-approach patients for a second blood sample.

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