Study protocol

Title: Characterisation of COVID-19 long-term immunity

RESEARCH REFERENCE NUMBERS

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BACKGROUND

In 2019 a novel coronavirus-induced disease (COVID-19) emerged in Wuhan, China. Shortly after its emergence, the Chinese Center for Disease Control and Prevention identified a new beta-coronavirus (SARS-CoV-2) as the aetiological agent. The clinical infection resulting from SARS-CoV-2 is known as coronavirus-induced disease (COVID-19) and is responsible for a global pandemic. The spectrum of disease resulting from COVID-19 is broad, with many individuals being asymptomatic or experiencing mild symptoms, whilst others require hospital-based care.

Currently, very little is understood about how SARS-CoV-2 interacts with the immune system. Immunological memory describes the ability of the adaptive immune system to mediate a response on second exposure to the same pathogen, which is more rapid and greater in magnitude than the response to initial pathogenic encounter. If robust immunological memory is not maintained following recovery from viral infection, then the host can be re-infected with the same pathogen multiple times during their lifetime. It is unclear whether robust long-lived immunological memory is established after recovery from COVID-19 or how this is influenced by factors such as age, co-morbidities, disease severity and hyperinflammation. It is essential that we characterise the SARS-CoV-2 specific memory response and understand how inflammation that may occur following infection both acutely and chronically influences the generation of immunological memory. In addition, very little data exist on the immunological career of patients in convalescence, particularly how this might vary by age and through epi-phenomena, such as the increasingly described “long-COVID”.

Reducing COVID-19 Disease

To reduce disease burden and associated mortality and morbidity from COVID-19, multiple interventions and developments are required including public health measures reducing transmission and protecting individuals at risk. The development of successful therapies, including vaccines, is dependent on detailed understanding of how the immune system responds to infection with COVID-19. However, as a newly emergent human pathogen, little is understood about the effect of COVID-19 infection on the immune system.

COVID-19 Interaction with the Innate Immune System

Innate immune sensing serves as the first line of antiviral defence and is essential for immunity to viruses. It seems likely that SARS-CoV-2 virus-host interactions will be similar to other coronaviruses (CoVs). Following activation, Rig-like receptors and toll-like receptors induce signalling cascades, leading to the phosphorylation of transcription factors (e.g. NF-kB) resulting in transcription of IFN and proinflammatory cytokines. CoV proteins inhibit several steps of signal transduction pathways that bridge the receptor subunits to STAT proteins activating transcription, preventing signalling downstream of IFN release. CoVs may actively promote other inflammatory pathways, contributing to pathology and these proinflammatory processes are likely contribute to the ‘cytokine storm’ observed in COVID-19 patients. Elevated systemic levels of the proinflammatory cytokine IL-6 have been reported in several COVID-19 patient cohorts and shown to correlate with disease severity. Increased IL-6 can also be associated with higher levels of IL-2, IL-7, IFN-ɣ, and GM-CSF, as seen in secondary hemophagocytic lymphohistiocytosis.

Evidence also suggests dysregulated myeloid responses may drive the COVID-19 clinical disease phenotype, such as acute respiratory distress syndrome (ARDS), cytokine release syndrome and lymphopenia. Neutrophil NETs (neutrophil extracellular traps) and macrophage crosstalk can drive later-stage inflammatory cascades, with data suggesting IFN responses may be pathological in later disease. Natural killer (NK) cells may also play a role in the immune response to COVID-19, as triggering NK cell activation may not only contribute to the resolution of infection, but also to the cytokine storm in ARDS. However, the mechanistic role played by lung-resident and recruited granulocytes in SARS-CoV-2 control and pathogenesis remains undefined.

Early data have highlighted some mechanisms by which the innate immune system responds to SARS-CoV-2 infection; however, the full duration and nature of these processes remains elusive. There is therefore an urgent and pressing need to accurately define these processes and the relative contribution that they make to individuals with COVID-19 infection and associated 'long-COVID' or Kawasaki-type phenomenon. However, it is likely that any activation of the innate immune system would be relatively short-lived. As such, this protocol will undertake sampling from participants with confirmed SARS-CoV-2 infection and obtain peripheral blood leucocytes and serum factors to assess the duration and nature of the innate immune response throughout the study duration.

T-cell Mediated Immunity

T-cells play a fundamental role in viral infections. For example, CD4 T-cells provide B-cell help for antibody production and orchestrate the response of other immune cells, whereas CD8 T-cells kill viral infected cells to reduce the viral burden and are important in viral clearance. In addition, dysregulated T-cell responses can result in immunopathology and may contribute to the development of severe disease. It is imperative that we characterise the T-cell response to SARS-CoV-2 and define whether robust long-lived T-cell memory is established following recovery from COVID-19 infection. It is also important that we understand how the T-cell response and subsequent formation of T-cell memory is influenced by factors such as age and different clinical phenotypes.

In acute SARS-CoV-2 infection, lymphopenia may occur with drastically reduced numbers of CD4 and CD8 T-cells, with some evidence that the degree of lymphopenia correlates with COVID-19 disease severity and mortality. Currently, little is known about specific phenotypic and/or functional T-cell changes associated with COVID-19 and whether boosting or limiting T cell activation would represent candidate therapeutic options to target hyperinflammation.

To facilitate an in-depth analysis of the phenotypic and functional properties of SARS-CoV-2-specific T-cells it is important to identify the SARS-CoV-2 epitopes/antigens that are targeted by effector and memory T-cells, as well as their immunodominance across a number of patients that express different chosen HLA molecules. While initial bioinformatic and experimental work has given some evidence of the major protein targets of the T-cell response, the identity of the immunodominant T-cell epitopes remains to be defined. This knowledge is critical for the design of vaccines that aim to elicit responses in both B and T-cell arms of the adaptive immune system as well as for the evaluation of SARS-CoV-2 specific T-cell immunity after vaccination and during natural infection.

This protocol therefore proposes a structured sampling framework over 9 months with participants recruited at different time points following SARS-CoV-2 infection. At each visit, peripheral blood mononuclear cells will be extracted from participants. The immune profile of T-cells will be analysed to determine the nature and duration of T-cell immune responses following SARS-CoV-2 infection. By defining this response, and the protein targets of the T-cell response, this study will directly contribute to the understanding of the immune response against SARS-CoV-2. The results from this study can be directly compared to T-cell responses in individuals who receive any future COVID-19 vaccination, and thus this study will act to provide a comparator for the efficacy of immunological responses in vaccinated individuals.

B-cell Mediated Immunity

B-cell responses to viral infections serve to protect from the initial challenge, and to provide extended immunity against reinfection. After encountering antigen, activated antigen-specific B-cells proliferate and differentiate into either antibody secreting cells (ASCs, or plasma cells), or become long-lived memory B-cells. Recombination of VDJ genes and somatic hypermutation of activated B-cells committed to become memory B-cells, leads to the generation of diverse B-cell repertoires with higher affinity for target antigens. Plasma cells formed during the acute and convalescent phases continue to secrete antibodies, giving rise to serological memory following acute infection. Memory

B-cells formed during the primary infection constitute the second arm of B-cell memory. Memory B-cells respond quickly to reinfection by generating new high-affinity plasma cells. Long-term protection is achieved through the induction of both long-lived plasma cells and memory B-cells. The activation and differentiation of virus specific B-cells during and after infection impacts the functional quality, magnitude and longevity of antibody responses and the capacity to response to re-infection. Memory B-cell responses to other CoVs, including SARS-CoV-1, are relatively short-lived, which may explain why immunity to these infection wanes over the period of a few years. As such, it is imperative that we describe in detail the B-cell response to SARS-CoV-2 in samples from different patient groups.

We will therefore take sequential samples from study participants with confirmed SARS-CoV-2 infection throughout the study, describing the nature and duration of the antigen-specific B-cells. This protocol will allow researchers to determine the type of differentiation of antigen-specific B-cells into antibody secreting cells and memory B-cells and describe the duration of this memory B-cell response. The results of this analysis will provide critical insight into the duration and nature of the immune response to SARS-CoV-2, enabling assessment of future potential vaccines and their implementation. Furthermore, this data will allow accurate modelling of global SARS-CoV-2 transmission dynamics and assessment of non-pharmaceutical interventions.

Antibody Responses

Humoral immune responses to coronaviruses are a particularly important area of study as neutralising antibody is believed to be the principle mode of immune-mediated protection to reinfection, as well as a likely mode of clearance in primary infection. Recently published peer reviewed articles and non-peer reviewed preprints indicate that most individuals infected with SARS-CoV-2 (as determined by RT-PCR) develop detectable antibody responses between 10- and 20-days post symptom onset. There have been mixed findings regarding the severity of symptoms and the level of antibody responses, with some studies showing no relationship between case severity and levels of antibody.

It is important to consider a potential role for pre-existing cross-reactive antibodies due to prior infection with other common human CoVs (which cause the common cold) on the outcome to SARS-CoV-2 infection. Studies exploring the role of cross-reactive antibodies to other CoVs suggest that there is some degree of protection provided by antibodies raised against heterogenous CoVs within the same family (i.e. alpha or betacoronviridae).

Importantly, the longevity of antibody responses and the level of neutralising antibody, is not known. Deciphering the range relationship between the severity of infection and longevity of protective humoral immune responses is crucial for predicting future outbreaks and the future relationship we will have with SARS-CoV-2; many studies assuming a short lived antibody responses (as is the case with other common CoVs) point towards SARS-CoV-2 becoming a seasonal respiratory virus.

Currently the duration, type and efficacy of antibody response to SARS-CoV-2 remain unclear. There is thus a pressing need to define the antibody response, as this will provide a critical insight into the immune response to SARS-CoV-2, enabling the assessment of potential vaccines and implementation of public health interventions. Understanding the duration of the antibody response to SARS-CoV-2 will enable more accurate modelling of infection in predicting future outbreaks and the likely future dynamics of SARS-CoV-2 infection

Rationale

It remains unclear as to whether long-lived robust immunological memory is formed after infection with SARS-CoV-2. As a novel human pathogen, there is little data to indicate how long immune memory persists for and in what proportion of patients it occurs. Following recovery from infection with many viruses, robust long-lived immunological memory responses occur and prevent re-infection from that virus. However, infection with some viruses does not stimulate sustained immunological memory and therefore it is possible to be re-infected with the same virus. Data

suggests that immunological memory formed following infection with other human coronaviruses may not be long lived. However, to date, there has been no study conducted to determine the magnitude, quality, and persistence of immune memory after SARS-CoV-2 infection.

The duration of immunity is critical to make informed decisions regarding the easing of public health control measures. For example, if immunity to COVID-19 is short-lived, there may therefore be increasing number of cases over the winter, which would help inform key decisions to refine and adapt the UK's public health control measures and ensure their feasibility for a prolonged period. In addition, we will generate data that is relevant to the development and implementation of vaccine programs.

It is also essential to establish how factors such as age, co-morbidity, disease severity, the occurrence of hyperinflammation (e.g. Kawasaki-like disease) and persistent immune activation influence the degree of immunological memory established. It is crucial that these key questions are answered because this will enable:]

(1) accurate modelling of global SARS-CoV-2 transmission dynamics and the impact of non-pharmaceutical interventions,
(2) development of vaccines capable of stimulating protective immunity; and,
(3) optimal design of vaccine strategies (if/when vaccines become available) to achieve protection that is sustained, as it may or may not be necessary to vaccinate individuals who have previously been infected with SARS-CoV-2.

This work will define the duration, nature and efficacy of the immune response following SARS-CoV-2 infection. It is critical as it will help understand the immunological responses in individuals following SARS-CoV-2 infection by undertaking a longitudinal observational cohort study of participants and define the immunological memory response following SARS-CoV-2 infection.

METHODOLOGY

This is a longitudinal observational cohort study of individuals, seeking to define the immunological memory immune response following SARS-CoV-2 infection. Sequential observations and clinical samples will be taken from individuals who have had SARS-CoV-2 infection as determined by positive antibody response, regardless of disease severity and as determined by symptoms, government testing or antibody testing as part of ALSPAC. Individuals who have not had infection with SARS-CoV-2 will be recruited as controls, and these participants will undergo the same sequential observations and clinical sampling procedures. The samples obtained from this protocol will then be analysed to examine the immune response to SARS-CoV-2, to determine whether robust long-lived immunological memory to SARS-CoV-2 is established, how long it persists after recovery from COVID-19 and how this is influenced by a range of different factors and clinical phenotype.

This study will use the Avon Longitudinal Study of Parents and Children (ALSPAC – also known as Children of the 90s by participants and the public) to recruit participants. Between April 1991 and December 1992 ALSPAC recruited more than 14,000 pregnant women into the study and these women (some of whom had two pregnancies or multiple births during the recruitment period), the children arising from the pregnancy, and their partners have been followed up intensively over two decades and we hold detailed historical data and biological samples for these participants with consent for use in future research. The original study mothers and their partners are referred to as the G0 cohort. The original offspring and now their partners are referred to as the G1 cohort.

Participant Selection

ALSPAC have undertaken 5200 serological SARS-CoV-2 tests on G0 and G1 cohort participants and administered questionnaires to participants during the pandemic. In order to achieve the research objectives outlined in this protocol, in each clinic round this study will aim to recruit:

- All G1 individuals and a comparator sample of G0 individuals meeting eligibility criteria who meet the study definition of cases
- Up to 200 G1and G0 individuals meeting eligibility criteria who meet the study definition of controls

Information from these along with linked NHS medical records data will be used to identify potential participants. Based on seroprevalence estimates, ALSPAC cohort size, and estimations of typical response to similar studies, this study therefore estimates recruiting approximately:

- 100 cases of previous SARS-CoV-2 infection at Visit 1 (approx. upper limit of 250 cases)
- 100 control individuals without previous SARS-CoV-2 infection age, sex and symptom matched to cases and 100 random controls without previous SARS-CoV-2 infection (approx. upper limit of 250 controls)

Where needed (i.e. if there are large numbers of potential cases), priority will be given to participants with pre-pandemic measures of BMI and lung function (objectively assessed at the age of 24 years) and previous biological samples.

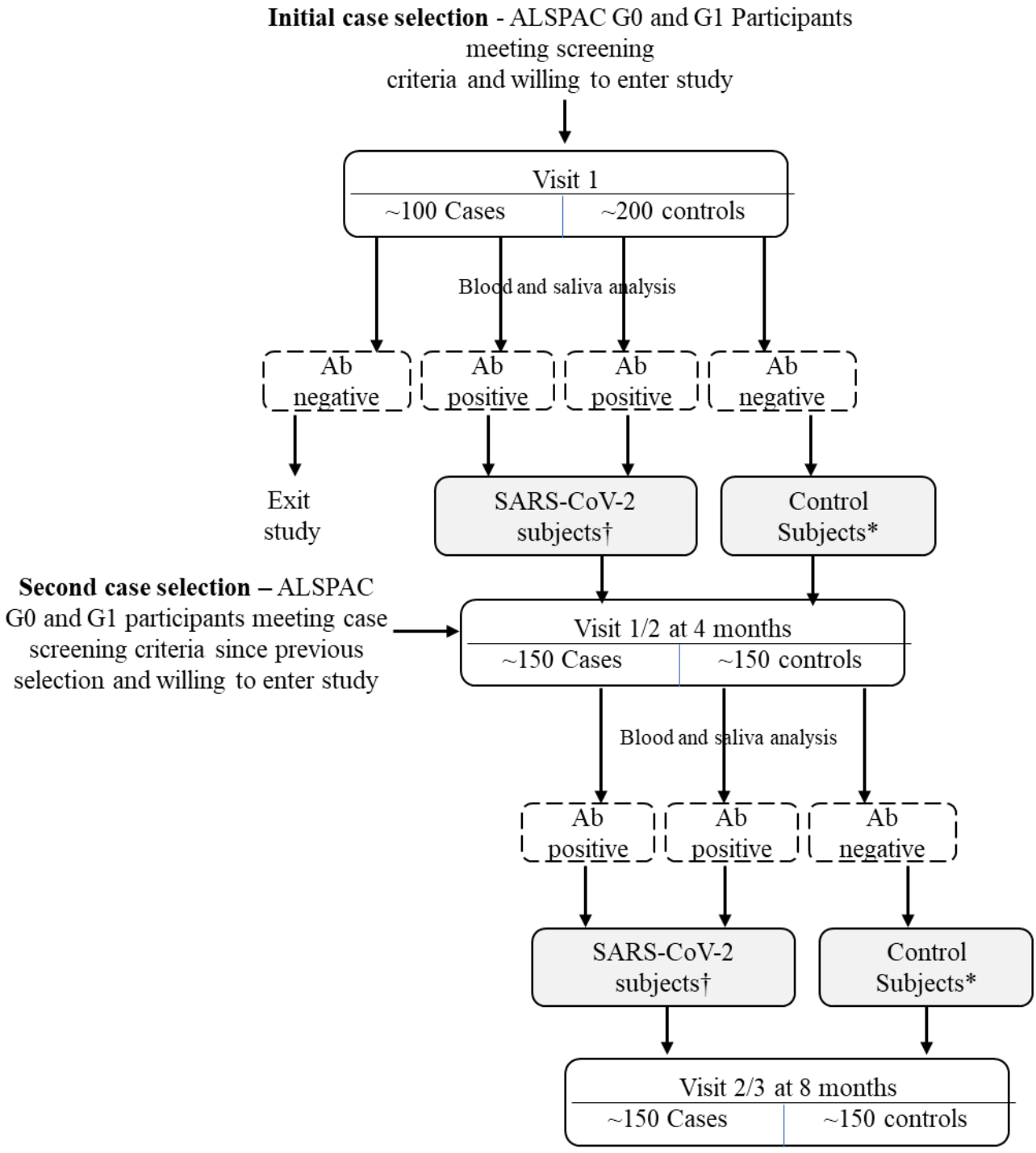
As part of an initial visit, blood will be taken and the antibody and T-cell response to SARS-CoV-2 determined. Individuals who are cases with a SARS-CoV-2 antibody response and/or a positivie T-cell response will be enrolled in the SARS-CoV-2 arm. Individuals who are cases with information available that there is no detectable SARS-CoV-2 antibody or T-Cell response will be considered seronegative and will not be required to participate further in the study. Individuals who are recruited as controls and have a confirmed SARS-CoV-2 antibody negative response will be enrolled into the control arm for two remaining visits, sufficient negative controls will be recruited to allow for the possibility that some controls will become infected with SARS-CoV-2 during the duration of the study and will not be eligible to complete all four visits. Those who were initially recruited as controls

who have subsequently tested positive will also be retained in the study and will move from control arm to case arm as appropriate. Where cases are no longer available, but new cases are defined, sample size will be maintained by the recruitment of new case events.

Pilot study

A pilot study will be undertaken on up to 10 healthy volunteers, who will be recruited on the basis that they are willing to participate in the pilot and have neither a clotting disorder nor are taking any blood thinning medication. These individuals will be asked to undergo the procedures outlined in this protocol including infection control measures, informed consent, blood sampling (4x10ml EDTA, 1x5ml Serum), saliva sample, physical measures, respiratory function testing, urine sample and a questionnaire. The pilot will be undertaken in order to ensure that operational and research standards are sufficiently operational before commencing the finalised study.

Study Flow Diagram



• Control participants met control screening eligibility criteria
† SARS-CoV-2 participants met study eligibility criteria

Primary Objective

To determine and describe SARS-CoV-2 specific humoral and cellular responses longitudinally and determine the magnitude and duration of immunological memory following COVID-19 infection.

Secondary Objectives

The secondary outcomes are:

(1) To determine and describe the nature, specificity and long-term persistence of the SARS-CoV-2 specific antibody response

(2) To determine how the SARS-CoV-2 specific antibody response relates to clinical phenotype

(3) To determine and describe the nature, specificity and kinetics of the B-cell memory response following COVID-19 infection, and in relation to clinical phenotype

(4) To identify key B-cell and T-cell epitopes to enable functional analysis of the SARS-CoV-2 memory response, and inform vaccine design

(5) To determine the magnitude, breadth, functional phenotype, and persistence of the T-cell response

(6) To describe how the memory T-cell response to SARS-CoV-2 relates to clinical phenotype of COVID-19 infection

(7) To describe and determine the effect of SARS-CoV-2 infection on components of the innate immune system, including:

a. inflammation-related serum proteins (e.g., complement, C-reactive protein, lectins such as mannose-binding lectin, and ficolins).

b. phagocytes and cells that release cytokines and inflammatory mediators.

c. surface and phagocyte granule antimicrobial peptides.

d. cell receptors that sense microorganisms and signal a defensive response (e.g., Toll-like receptors); and,

(8) To determine and describe the impact of hyperinflammation and chronic immune activation on the generation of long-term immunity

(9) Assess prevalence of cross-reactivity against circulating CoVs and how this might influence the outcome of response to COVID infection.

(10) Assess changes in the exercise capacity test, SF36 and self-reported dyspnoea during the convalescent period.

Study design

This is a longitudinal observational cohort study of adults within the Bristol area, conducted on individuals who may fall into the following groups of patients:

(1) healthy seronegative controls (some of whom may become infected during the study),
(2) asymptomatic/pauci-symptomatic
(3) symptomatic infection not requiring hospitalisation,
(4) moderate illness not requiring hospitalisation,
(5) hospitalised patients who did or did not require mechanical ventilation

Sampling framework

Current estimates of COVID-19 prevalence in the SW England (based on seroprevalence data) range between 2-6%, with age and time since infection contributing to differences in patient groups. It is therefore reasonable to assume that seroprevalence is approximately 3%. ALSPAC have undertaken 5000 serological SARS-CoV-2 tests on cohort participants; therefore, if only 3500 of these are returned, we would expect to identify 100 individuals seropositive to SARS-CoV-2. If only 50% of these 100 individuals wish to enter into this study, the cohort of seropositive individuals would be 50.

The number of seropositive individuals will be increased by incorporating ALSPAC cohort individuals who:

1. self-report as being highly likely to have had COVID-19 infection (from either positive SARS-CoV-2 PCR test performed at NHS care facility, COVID-19 testing site or home testing), **OR**
2. have been told by a physician that, in the opinion of that doctor, they have had a clinical illness likely to be COVID-19 **OR**
3. based on a combination of symptoms they are likely to have been cases

DATA COLLECTION

Cases - inclusion criteria

This study will be undertaken on participants of the ALSPAC cohort meeting the following criteria:

1. Aged 25 or over, **AND**
2. Individuals who have undergone SARS-CoV-2 antibody testing through the ALSPAC SARS-CoV-2 serology study (IRAS 289493), **OR**
3. Individuals who self-report or through linkage to official health records as being highly likely to have had COVID-19 infection (from either positive SARS-CoV-2 PCR test performed at NHS care facility, COVID-19 testing site or home testing), **OR**
4. Individuals who have been told by a physician that, in the opinion of that doctor, they have had a clinical illness likely to be COVID-19.

Control participants – inclusion criteria

1. Aged 25 or over, **AND**
2. Individuals who self-report as not experiencing symptoms of COVID-19 via the symptom survey and do not have case status through linkage to official health records, **AND**
3. Self-report as not having had positive SARS-CoV-2 molecular test, **AND**
4. Are confirmed SARS-CoV-2 seronegative at Visit 1 and then at visit 2

Exclusion criteria for both controls and cases

1. Do not wish to participate in this research study
2. Participants taking blood thinners (e.g. warfarin) or with a known clotting disorder
3. Do not meet eligibility criteria for respective arm

Invite procedure

An initial invitation email (*Initial invite v2*), and link to a participant information sheet (*Information Sheet v2)* will be sent to eligible participants, via contact details held, with participant consent, in the ALSPAC contact management system. Appointments will be offered on a range of days and times, including weekends. Participants will be able to respond by telephone or email. A reminder email will be sent if there is no response after one weeks (*Reminder email v2*) and if they do not respond after a further week they will be called by a member of the ALSPAC research team. This is an invitation procedure used previously within ALSPAC and study participants are familiar with contacts from the study by these methods.

Following the initial visit, samples will be analysed for current serological response. Those cases who are seropositive or T-Cell positive at Visit 1 and all controls whether confirmed as seronegative or seropositive will be invited (*follow up invite v2*) to complete repeat visits 4 months, and 8-12 months post visit 1. Those not eligible to continue the study will be notified by email (*follow up no visits v2*).

Where cases are no longer available, but new cases are defined, sample size will be maintained by the recruitment of new case events.

Before the clinic visit - (Visits 1-4)
Once booked for an appointment, the participant will be sent a confirmation email (*Confirmation letter v1, with a link to visiting children of the 90s COVID-19 information sheet*) and then an appointment reminder call the day prior to their appointment this will include a check for any current COVID-19 symptoms.

At the clinic visit
The order of measures is as follows:

1. Informed consent
2. Blood sampling (4x10ml EDTA, 1x5ml Serum)
3. Saliva sample
4. Physical Measures (Height, weight)
5. Respiratory function testing
6. Urine sample
7. Questionnaire

Infection Control Measures
We will ensure that participants:

- are advised to arrive at Oakfield House already wearing a face covering. Masks will be provided where they forget.
- are supplied with additional PPE where required
- remain more than 2 metres away from anyone who does not live in the same household, wherever possible,
- participants who are symptomatic will be asked to not attend and amend their appointment
- participants will follow 14-day household quarantine rules if someone they live with becomes symptomatic, and be asked to reschedule study visits
- employ good hand hygiene, following advice and appropriate thorough handwashing

In accordance with guidance from the Department of Health & Social care, any Fieldworker working within 2 metres of a patient with current confirmed or suspected COVID-19 will wear an apron, gloves, a surgical mask and eye protection. As study participants are individuals with either possible or confirmed previous COVID-19 infection or controls who are confirmed SARS-CoV-2 negative after Visit 1, Fieldworkers and other research staff will wear facemask, gloves and aprons.

Study visits will occur in a location that is approved for clinical research which is not located within an acute medical care facility, thereby reducing the exposure risk for research staff and study participants to individuals with SARS-CoV-2 infection. All Fieldworkers and research participants attending this research study will have their temperature taken on arrival as screening for asymptomatic COVID-19 infection. Should any participant or Fieldworker have an elevated temperature, they will be advised to return home, follow current guidelines regarding isolation and testing, and study visits will be rescheduled.

Informed Consent
All individuals are required to complete a consent form (*Consent form v1*) for various aspects of biological sampling and physical measures. All samples and measures are voluntary, and this is emphasised at every opportunity and they can be stopped at any time.

When the participant arrives, they will be greeted and taken to a private room and the Fieldworker will:

- Check the participant's personal details – name, address, date of birth – are correct.
- Record on REDCap data collection system that participant has arrived and that personal details are correct on REDCap data collection system.
- Ask the participant if they have received the participant information sheet and had time to read it
- Explain the physical measure procedures and the biological samples to be taken to the participant
- Ask if they have any questions
- Show the participant the electronic consent form and ask them to read it
- Ask if they have any questions about the consent form
- Ask them to electronically sign consent form (*Consent form v1*)

Physical Measurements

The Fieldworker will measure and record the height and weight of the study participants.

Blood Sampling

4 x 10ml EDTA tube and 1 x 5ml serum tube will be taken, with the participant's consent.

Blood samples will be processed, and plasma, serum and peripheral blood mononuclear cells (PBMCs) extracted and stored by the Bristol Bioresource Laboratories. Researchers can apply to use the biological samples collected at this visit and any associated material from processing via the ALSPAC Executive. All samples, electronic data and human tissue is stored securely on site at Oakfield House. The Bristol Bioresource Laboratories within Oakfield House is licensed by the Human Tissue Authority for long-term storage of samples. Blood samples will be taken in a room in which required equipment and adequate stocks of necessary consumables will be kept. The required number of sets of syringes, tubes, cotton wool etc. will be set up in the blood taking room prior to each clinic setting.

The Fieldworker will:

- Put on PPE according to COVID-19 risk assessment and supply the participant with any additional PPE required
- Ask the participant if they have ever had a blood sample taken before and if there were any problems
- Ask the participant if they are allergic to plasters
- Explain blood taking procedure to the participant
- Confirm electronic and verbal informed consent (reminding the participant that taking part is voluntary and that they can stop or withdraw at any time)
- Ask the participant if they prefer which arm blood is taken from. If they have no preference suggest the non-dominant arm (left if they are right-handed and right if they are left-handed)
- Ask the participant to lie down on the couch, with the antecubital fossa of which arm blood will be taken from exposed.
- Place tourniquet around participant's upper arm and gently tap over veins to make them prominent
- Explain to the participant that they should say if they start to feel faint, sick or experience pain beyond what they expect during blood taking (stop if any of these occur)
- When a suitable vein has been located, explain to the participant that you are now going to take the sample and that they will feel the needle prick.
- Clean the site where sample will be taken with alcohol swabs
- Take blood samples using vacutainer system
- Loosen tourniquet as soon as blood is being obtained

- Place cotton wool ball over needle entry and gently withdraw needle
- Press firmly on cotton wool ball
- Explain to participant the sample has been completed and ask him/her to press firmly on cotton wool ball over puncture site
- Gently shake tubes as necessary
- Store samples ready for collection by laboratory technician (3x EDTA stored at RT, 1xEDTA and serum stored on ice)
- Dispose of needle and syringe in sharps bin and all other consumables requiring sterile disposal (cotton wool balls with blood staining) in yellow sterile waste bin
- Ask participant how they are feeling. Suggest they rest for a few minutes on the couch
- Check puncture site and cover with plaster as necessary
- Ask participant to slowly sit up
- Record the number and type of each tube successfully filled to required amount, on the REDCap system

Saliva Sample

The Fieldworker will label a green saliva collection tube (cryovial) with a barcode label of the participant's study number.

The Fieldworker will:

- Will move to 2m distance from the participant
- Ask the participant to put on a pair of non-sterile gloves
- Open the screwcap lid of a green sample tube (green)
- Hold the open tube upright in one hand and place the funnel into tube using other hand
- Then spit into funnel until saliva reaches between the 1.0 and 1.5 mark of the tube
- Put the saliva funnel in small clear plastic waste sachet
- Replace the screwcap lid and place the specimen in a clear plastic bag
- Seal the plastic bag
- Remove their gloves and wash their hands with soap and water for 20 seconds

Respiratory function testing

The Fieldworker will ask participants to undertake tests to evaluate the exercise tolerance and functional capacity of study participants. This evaluation can be undertaken by either the 1-minute sit-to-stand test (STS), or alternatively the 6-minute walk test (6MWT)

1-minute STS:

A standard chair (height 46–48 cm), with flat seat and no arm rests will be used.
The Fieldworker will:

- Explain 1-minute STS procedure to the participant
- Ask the participant if there is any reason why they feel they may not be able to complete the test, or if they wish to undertake this procedure
- Ensure that the chair is positioned against a wall
- The participant will be asked to sit in the chair
- Check that the participant is sitting with knees and hips flexed to 90°
- Attach a pulse oximeter to measure oxygen saturations and heart rate
- Record the resting pulse oximetry and heart rate
- Ensure that the participant's feet placed flat on the floor hip-width apart, and the hands placed on the hips
- Give the participant the following instructions:
    - The purpose of the test is to assess your exercise capacity and leg muscle strength. The movement required is to get up from this chair with the legs straight and sit back continuing the repetitions as fast as possible within one minute. I will give you the

countdown '3, 2, 1 Go' as an indication to start and also, I will tell you when we are at the 15 remaining seconds. If required, you can make a break and resume the test as soon as possible.

- A stopwatch will be used to time 60 seconds
- Validate each rise to check if complete sit-to-stand-to-sit sequence was achieved
- Count the number of rises achieved within the 60 seconds
- If during the procedure, the oxygen saturations dip below 85%, the procedure will be stopped.
- The lowest recorded oxygen saturation and highest heart rate will be recorded.
- The oxygen saturation and heart rate at the end of the test will be recorded.

6MWT:

The Fieldworker will ask the participant to undertake a 6MWT if they are unable to undertake the STS test. The 6MWT was developed by the American Thoracic Society, officially introduced in 2002 and there are comprehensive guidelines for its usage.

Equipment needed: Stopwatch, measuring/trundle wheel to measure distance covered, 30-metre stretch of unimpeded walkway, two cones to mark the distance that needs to be covered, pulse oximeter for measuring heart rate and oxygen saturations.

The Fieldworker will:

- Provide the following instructions:
    - The object of this test is to walk as far as possible for 6 minutes. You will walk back and forth in this hallway. Six minutes is a long time to walk, so you will be exerting yourself. You may get out of breath or become exhausted. You are permitted to slow down, to stop, and to rest as necessary. You may lean against the wall while resting but resume walking as soon as you are able. You will be walking back and forth around the cones. You should pivot briskly around the cones and continue back the other way without hesitation. Now I am going to show you. Please watch the way I turn without hesitation
- Then demonstrate the 6MWT
- Attach a pulse oximeter to the participant
- Record the baseline pulse rate and oxygen saturations
- The participant will be asked to start the 6MWT
- The nurse will provide this standardised encouragement during the test:
    - After the 1st minute: "You are doing well. You have 5 minutes to go."
    - When the timer shows 4 minutes remaining: "Keep up the good work. You have 4 minutes to go."
    - When the timer shows 3 minutes remaining: "You are doing well. You are halfway done.
    - When the timer shows 2 minutes remaining: "Keep up the good work. You have only 2 minutes left.
    - When the timer shows 1-minute remaining: "You are doing well. You only have 1 minute to go.
    - With 15 seconds to go: "In a moment I'm going to tell you to stop. When I do, just stop right where you are, and I will come to you."
    - At 6 minutes: "Stop"
- If the participant stops at any time prior, the Fieldworker may say, "You can lean against the wall if you would like; then continue walking whenever you feel able"
- Not use other words of encouragement (or body language) to influence the patient's walking speed.
- Accompany the participant along the walking course but keep just behind them. The nurse will not lead them.

- Record the total distance walked; oxygen saturations and heart rate at completion of 6MWT; lowest oxygen saturations and highest heart rate.
- If the oxygen saturation drops below 85% the test will be stopped.

Computerised questionnaire

Participants will be asked to complete a short questionnaire during their visit. This questionnaire will be filled in as a computerised questionnaire on the REDCap data collection system. Prior to commencement of the computerised questionnaire session the Fieldworker will:

- Describe the computerized questionnaire included in the session and how long the session is likely to take
- Provide the participant with the opportunity to ask questions
- Answer any questions that the participant may have
- Set-up the participant, on the computer for their use.

The questionnaire will include the Short Form (36) Health Survey (SF-36) assessment and an assessment of their breathlessness (dyspnoea) by the Modified Borg Dyspnoea Scale (MBS). At visits 2 and 3, it will include questions about symptoms, tests and vaccinations since the previous.

SF-36

The original SF-36 stemmed from the Medical Outcome Study, and it is a 36-item, patient-reported survey of patient health. The SF-36 is a measure of health status. is commonly used in health economics as a variable in the quality-adjusted life year calculation to determine the cost-effectiveness of a health treatment.

Modified Borg Scale (MBS)

The MBS is a 0 to 10 rated numerical score used to measure dyspnoea (breathlessness) as reported by the patient during submaximal exercise and often administered to patients before and after the six-minute walk testing (6MWT). Participants will be asked to grade the severity of their dyspnoea at rest and during activity.

Revised Borg Scale for Grading Severity of Dyspnea
0 - Nothing at all
1 - Just noticable
2 - Very slight
3 - Slight
4 - Slight-moderate
5 - Moderate
6 - Some difficulty
7 - Moderately severe
8 - Sever
9 - Very sever
10 - Panic level, maximal shortness of breath

COVID-19 Symptoms, tests and vaccinations

A short series of questions will be asked about any symptoms experienced since the last visit, these questions are repeats of questions asked to ALSPAC participants on 3 previous occasions during the pandemic and will enable us to track convalescence.

A series of questions will be asked about likely exposure to COVID-19 in the 28 days prior to the clinic visit, this provides details required for completion of a risk assessment for the processing of the biological samples.

Participants will be asked if they have been vaccinated, when they received the vaccination and which vaccination they had. This will be vital in understanding results of analysis of the biological samples.

Use of existing ALSPAC samples in this study
ALSPAC has collected peripheral blood lymphocytes, blood, urine and saliva samples at previous research clinics (http://www.bristol.ac.uk/alspac/researchers/our-data/biological-resources/) The participants were asked to consent to "future use" when the samples were taken. Where appropriate consent was obtained for existing samples from the participants in this study cells and serum components may be used to establish a baseline immune phenotype or response pre-exposure to SARS-CoV-2 infection that is matched to individuals within the cohort. These cells and serum components will undergo the same analysis as research samples taken during this protocol.

PARTICIPANT FEEDBACK AND INCIDENTAL FINDINGS

The investigations performed in this study will not lead to incidental findings likely to impact on participants such that feedback to them or their healthcare providers is required. Furthermore, following processing of biological samples obtained, the laboratory analysis is likely to be undertaken at a time period after the specimens are obtained; hence, any results are not in real time for participants in this study Therefore, feedback to participants will not be provided.

In the event of any unexpected disclosure by study participants to the Fieldworker concerning their health status (for example struggling with activities of daily living or reduced exercise tolerance due to breathlessness), participants will be advised to consult their usual healthcare practitioner (e.g. General Practitioner) for further advice and ongoing treatment.

PROCESSING, TRANSPORT, ANALYSIS AND STORAGE OF BIOLOGICAL SAMPLES

Blood, urine and saliva samples will be taken from all consenting participants. Samples will be processed, and aliquots of samples stored for analysis. Samples will be labelled with barcoded ID numbers and personal information will not be passed to researchers analysing the samples. Samples will be stored for future use provided appropriate consent has been obtained. All samples will be used, stored and disposed of in accordance with the Human Tissue Act 2004. Oakfield House is licensed by the Human Tissue Authority for long term storage of samples.

- A laboratory technician will check the REDCap system which will indicate if samples have been taken. The potential blood samples that could be taken are 4 x EDTA tube and 1 x serum tube
- If samples have been taken the technician will collect from clinic. All tubes will be collected at room temperature. If REDCap does not show that samples have been taken within ½ an hour of the participant's appointment time the technician will contact the clinic team.
- On receipt of samples the technician will check that the barcode labels of the samples match the clinic ID on the REDCap system and that all samples taken in clinic match the samples received.
- Consent to individual analyses and storage for future genetic and non-genetic research will be checked. Samples will then be processed according to the consent given. If any consent data is missing the technician will contact the clinic team.
- PBMCs and/or PBLs will be isolated from blood taken in the 3 EDTA tubes which have been kept at room temperature using standard density gradient separation techniques. The sample processing procedure will commence within 3 hours of drawing blood. PBMCs and/or PBLs will be stored in liquid nitrogen and plasma will be stored at -80 ^{o}C.
- The 4th EDTA tube will be centrifuged and plasma aliquoted in 200ul and 500ul aliquots for storage at -80 ^{o}C. This is the ALSPAC standard protocol and will allow samples to be

comparable to those taken in other ALSPAC clinics. Provided consent was obtained for future genetic analysis then the buffy coat will be stored -80 ºC for future DNA extraction. The serum tube will be allowed to clot for 30 mins before centrifugation at 4 ºC. Serum will then be aliquoted in 200ul and 500ul aliquots for storage at -80 ºC.

- Samples may be sent to other labs both internal and external to the University of Bristol to undertake analyses. The Bristol Bioresource Laboratories will be responsible for the transport of samples from Oakfield House to other labs. For samples to be sent externally they will be shipped on dry ice with next day delivery to maintain sample integrity. A University of Bristol approved courier will be used that has a tracking system so that samples are traceable throughout delivery. The receiving laboratory will acknowledge receipt of samples upon delivery. A list of all samples to be analysed will be sent to the chief technician of the receiving lab, any discrepancies will be reported to either the BBL Chief Laboratory technician or Head of Biosamples. Any specific storage conditions will be adhered to by the receiving lab.
- PBMC/plasma or serum samples will be transferred to the School of Cellular and Molecular Medicine, Biomedical Sciences building, Immunology wing (F Floor) to Prof L. Wooldridge and Dr L.Rivino's laboratories (F60-F65). Samples will be stored and used as approved by the UoB Biological Safety Commitee (BGMSC activity #924; CBA1.18.20.1). PBMC samples will be stored in padlocked liquid nitrogen tanks (B floor, liquid nitrogen tank storage room), while plasma/serum samples will be stored in padlocked - 80ºC freezers (Prof L. Wooldridge/Dr L.Rivino's lab F65; Prof A.Finn's lab, B floor). The freezers/liquid nitrogen tanks are all located in access restricted areas accessible to authorized personnel only.
- Any remaining material following analysis will be returned to Bristol Bioresource Laboratories and stored in the ALSPAC biobank, this includes stocks of DNA and RNA.

LABORATORY ANALYSIS

Detection of SARS-CoV-2:

Saliva will be tested for SARS-CoV-2 using the reverse transcriptase polymerase chain reaction (RT-PCR) assay recommended by the World Health Organisation or adaptations thereof as the technique is refined and in conformity with Public Health England standard operating procedures. Briefly, after a viral inactivation step, RNA will be extracted from samples either manually or using an automated platform, reverse transcribed into cDNA, and stored at -70ºC. Samples will be prepared and real time PCR for SARS-CoV-2 will be performed using Thermo-Fisher QIAgility and QuantStudio or similar platforms. Viral detection will be expressed quantitively as cycle threshold (Ct) number for samples amplifying below the cut off for each assay.

SARS-CoV-2 specific serum and salivary antibodies:

ELISAs and Western Blots will be used to quantify titres of each antibody isotype (e.g. IgM, IgA, IgG) specific to SARS-CoV-2 viral proteins/epitopes being produced including (but not limited to) the spike glycoprotein, the receptor binding domain (RBD) and nucleoprotein. Techniques such as UREA based ELISA or surface plasmon resonance (SPR), will be used to determine the extent of antibody affinity maturation by calculating the avidity index of antibodies towards the spike glycoprotein and RBD over time (Mann et al. 2014). The neutralising activity of antibodies will be measured using techniques such as a high-throughput pseudovirus neutralization assay using SARS-CoV-2 spike protein pseudotyped lentiviral particles or SARS-CoV-2 microneutralisation assays with live virus.

Analysis of the B-cell response:

To describe the dynamics of the B-cell response we will enumerate and characterise antibody secreting cells (ASCs/plasma cells/plasmablasts), activated B-cells (ABCs) and memory B-cells

(MBCs) at various timepoints. This will be achieved using a combination of techniques which will include (although are not limited to) flow cytometry (via fluorescently labelled viral antigens and markers for ASCs, ABCs versus MBCs, etc.), B-cell ELISpots (Ellebedy et al. 2016; Clutterbuck et al. 2012) and antigen specific B-cell proliferation assays. The heterogeneity and degree of somatic hypermutation (SHM%) in SARS-CoV-2 specific B-cell responses will be determined by B-cell repertoire sequencing using RNA from PBMC and will identify 'public' versus 'convergent' B cells.

In-depth analysis of T-cell responses:

The SARS-CoV-2 specific T-cell response will be assessed in detail at various time points using a range of techniques These will include (but not be limited to) multi-parameter flow cytometry and activation assays such as ELIspots, ELISAs, proliferation assays and Intracellular Cytokine Staining (ICS) after stimulation with antigenic viral proteins and/or their peptide derivatives. In conjunction with these activation assays we will also perform in-depth phenotypic analysis of the antigen-specific T-cell response using multi-parameter flow cytometry. In addition, T-cells will be enumerated and directly characterized *ex vivo* from human blood using peptide-HLA (p-HLA) tetramers. Prior to some of these experiments we will need to determine the T-cell epitopes that can be recognized by the SARS-CoV-2 specific T-cell response. This will be achieved using a range of techniques such as MHCI binding algorithms, the analysis of previously identified SARS epitopes that are shared with SARS-CoV-2, overlapping peptide libraries and/or immunoproteomics. These candidate epitopes will be validated using patient samples and used to generate p-HLA tetramer reagents, and to perform in-depth phenotypic and functional analysis in conjunction with flow cytometry and T-cell activation assays. As such, for some experiments, it will be necessary to determine the HLA type of the patient or normal donor. In some instances, we may establish T-cell lines and/or T-cell clones that will be used to conduct in-depth analysis as described above, as well as combinatorial peptide library (CPL) analysis to assess T-cell cross-reactivity.

Immuno-phenotyping/Immune profiling (using serum/plasma and/or blood cells):

In-depth immune profiling will be performed at various time points. Flow cytometry will be used to characterise a range of immune subsets in collected samples from controls and different patient groups including those that display features of acute/chronic inflammation or immune activation. These will include: CD4 T cell subsets, CD8 and gamma-delta-T-cells, B-cells, NK and NKT-cells, monocytes, peripheral blood macrophages, as well as myeloid and plasmacytoid dendritic cells, neutrophils and platelets. Pro-inflammatory cytokines/chemokines and other soluble factors associated with the immune response will also be measured in the serum/plasma of the same patients.

Additional Assays

In addition to the assays described above we will be performing additional assays to assess the immune response to COVID-19. This may include techniques such as B-cell receptor (BCR) sequencing, T-cell receptor (TCR) sequencing, NanoString analysis, RNAseq, single cell analysis, transciptomics, proteomics, cell signalling analysis, analysis of cellular cytotoxicity for e.g. using XCELLigence RTCA systems etc. We will also apply the techniques described above to examine cross-reactive immune responses against other pathogens such as the circulating human CoVs, so that we can quantify T-cell, B-cell and antibody cross-reactivity and define how cross-reactivity may influence response to infection and/or vaccination. T-cell epitopes identified as described above will also be used to generate T-cell lines or clones that can be used for in-depth phenotypic and functional analysis (such as cross-reactivity against other viruses, TCR sequencing etc..) and generate T-cells with potential therapeutic benefit. SARS-CoV-2 specific T-cells will be assessed for reactivity against target cells such as SARS-CoV-2 infected cells to confirm whether they are SARS-CoV-2 specific.

Descriptive analysis of other measures

We will describe the cases (and controls) according to a number of pre-pandemic measures including gender, BMI, lung function, occupation and other sociodemographic measures. Changes over time

during the convalescent period will then be examined. This will focus on changes in self-reported measures of SF36 and dyspnoea and the objective respiratory function tests. Depending on the number of controls enrolled, we may be sufficiently powered to compare cases with controls in terms of the key outcomes.

DATA MANAGEMENT

Data will be collected on a computer-based system which is already in place and uses the REDCap software (http://www.project-redcap.org/). Any relevant research data will be extracted, cleaned and prepared for future use. All electronic data and human tissue are stored securely on University of Bristol premises.

Confidentiality:

Only anonymised data are shared with researchers. Strict procedures for collaboration are in place, which prevent collaborators linking data without our knowledge. Addresses, postcodes, e−mail addresses and telephone numbers are stored securely on separate servers to those which store the scientific data. Researchers with access to data files are denied access to the administration databases and vice versa. Descriptive analyses will be performed in-house

ADVERSE EVENT REPORTING

Risk assessments are carried out on all clinic measures and described in our risk management plan, and risks minimised via implementation of an action plan. Any adverse events will be recorded using University of Bristol incident reporting system – all incidents are thoroughly investigated, and root-cause analysis conducted.

Rigorous Health & Safety systems are in place with a series of SOPs available to deal with identified risks e.g. needle stick injuries.

COMPENSATION FOR PARTICIPANTS

Study participants will receive £10 as a thank you for each visit completed. This is based on what participants have previously received for taking part in similar activities within ALSPAC.

QUALITY CONTROL AND QUALITY ASSURANCE

The Fieldworker will be fully trained in the procedures ensuring specialist training where appropriate. Data collected on each participant will be recorded by a fully trained Fieldworker at the time of collection. The data collection system is computer based and has many data checks built into the system, including range checks and prompts for missing fields, thus minimising data entry errors. The investigators will be responsible for data quality

INSURANCE

The study is insured by the University of Bristol.

SPONSORSHIP AND ETHICAL ARRANGEMENTS

The University of Bristol is the research sponsor for this study.

This study will be reviewed by an NHS research ethics committee. Any amendments will be submitted for review to the sponsor and ethics committee prior to implementation.

The application has also been reviewed by the ALSPAC Executive, anonymous reviewers and grant board members for the European Research Council, the ALSPAC independent Law & Ethics Committee and the Children of the 90s original cohort advisory panel (OCAP).