Study Protocol

Full Title:	Investigation of the effects of dietary lecithin on intestinal permeability, bacterial translocation, microbiota and glucose metabolism
Study Acronym:	Food additives – do processed diets impact on gut and metabolic health (FADiets)
Sponsor:	University of Aberdeen
Funder:	Medical Research Council Grant MR/P023606/1
Local Principal Investigators:	Professor Alexandra Johnstone & Dr Alan Walker
Chief Investigator:	Prof B Campbell, University of Liverpool
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Internal study number:	810
ISRCTN / Clinicaltrialsgov No:	Clinicaltrialsgov ID: NCT03842514
Version Number and Date:	Version 4 18/02/2019

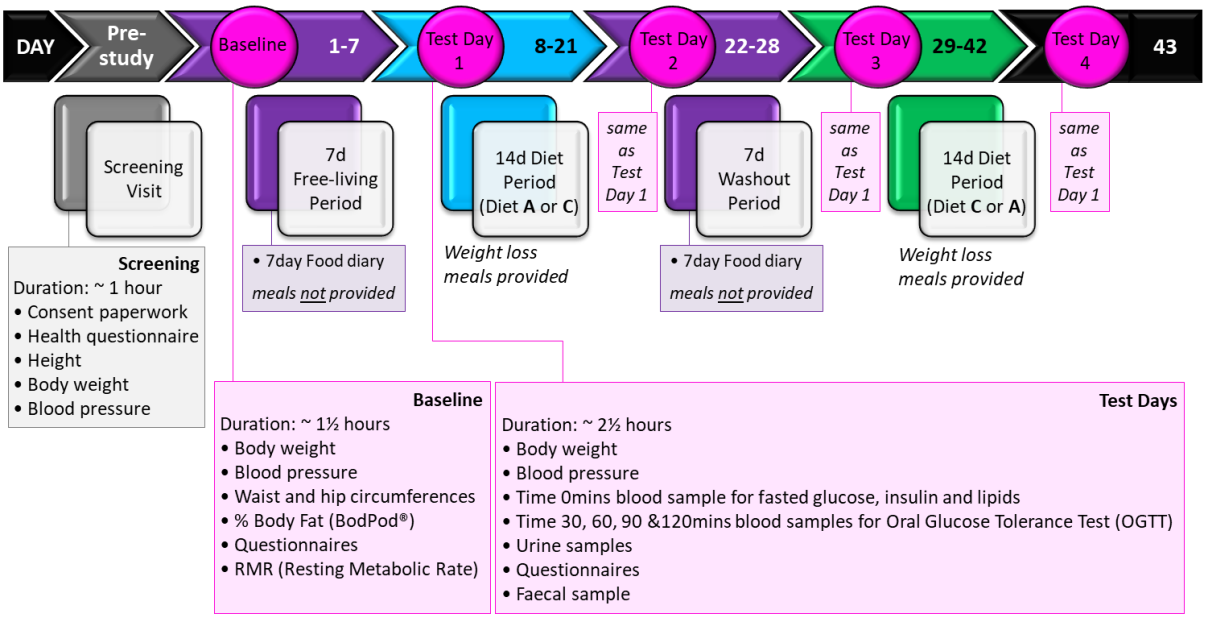
List of Abbreviations

AUC	Area under curve
CI	Chief Investigator
CRF	Case Report Form
GCP	Good Clinical Practice
ISF	Investigator Site File
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
OGTT	Oral glucose tolerance test
SCFA	Short-chain fatty acid
SOP	Standard Operating Procedure
TMF/SMF	Trial/Study Master File
UoA	University of Aberdeen
UoL	University of Liverpool
VOC	Volatile Organic Compound

Protocol Summary

A dietary intervention study is proposed to investigate the effect, in healthy human volunteers, of dietary lecithin (soy lecithin), a commonly used/consumed emulsifier, on markers of gut function particularly bacterial translocation (assessed by measure of venous blood bacterial DNA, circulating lipopolysaccharide [LPS] binding protein and soluble CD14), gut inflammation (assessed by measurement of faecal calprotectin), gut microbiota activity/composition (faecal short-chain fatty acid [SCFA] profile and bacterial diversity [16S ribosomal RNA genes]) and glucose metabolism (measured by oral glucose tolerance test [OGGT], plasma fasted lipids and insulin).

Protocol Summary Diagram



1.0 Introduction

Food additives are used for a range of functions in food including:

- To maintain the nutrient composition of the food and to keep it safe to eat
- To make food look or taste better
- To extend the shelf and storage life of a food product
- To improve the nutritional composition of a product (e.g. increase the vitamin content e.g. by adding ascorbic acid)
- Aiding in the processing and manufacture (e.g. emulsifiers, to help mix together ingredients)

This study is interested in the emulsifiers that help mix together ingredients like oil and water that would normally separate, commonly found in mayonnaise. All additives are thoroughly assessed for safety before they are permitted for use, and they are only then permitted to be used in a limited range of products and in certain amounts. Most additives are only permitted to be used in certain foods and are subject to specific quantitative limits. Lecithin is given a number of E322 listed in the FSA website https://www.food.gov.uk/business-guidance/eu-approved-additives-and-e-numbers

1.1 Background literature

Recent research, initially performed on cultured cells and tissues in the laboratory [1] and subsequent studies in mice [2], suggest that some permitted food emulsifiers may cause an increase in bacterial translocation across the gut lining, and/or alterations in the microbiota, with consequent low-level inflammation and altered glucose metabolism. These studies have been performed using a synthetic food emulsifier known as Polysorbate 80, as well as carboxymethylcellulose.

Use of synthetic emulsifiers in foods is however considerably exceeded by the use of natural lecithin, typically sourced from egg or soy, but also consumed in the normal diet in milk or meat. The average diet contains about 1 to 5 g of lecithin per day (WHO [3]). Lecithins contain varying amounts of the phospholipids: phosphatidyl choline (egg lecithin 66 – 80 %, soy c33%), phosphatidyl ethanolamine (egg lecithin c12%, soy 20-30%) and phosphatidyl inositol (egg lecithin c5%, soy c20%) [4, 5]. A single egg yolk contains approximately 1.8g lecithin [4]. In experimental situations, much larger intakes have been studied in man (22 to

83 g per day for 2 to 4 months) without untoward effects and it has not therefore been considered necessary to define a safe limit (WHO [3]).

1.2 Rationale for the study

This study primarily aims to determine whether short term (two week) high intake of lecithin alters gut function, as indicated by increased presence of bacterial DNA, soluble CD14 or LPS binding protein in the circulation (venous blood sampling), increased gut inflammation (faecal sampling for white blood cell components such as calprotectin), gut microbiota activity (faecal SCFA and bacterial DNA profile including estimation of diversity) and altered glucose metabolism (assessed by oral glucose tolerance test). This is achieved by comparing a low-emulsifier diet with a diet with soy lecithin emulsifier added – all food is provided to subjects as a controlled diet trial.

The Research Question

Does dietary intake of soy lecithin alter the intestinal lining and the microbes that normally exist in the intestinal lumen, in healthy subjects, consumed over a two-week period (in comparison to a low-emulsifier diet)?

2.0 Study objectives and outcomes

<u>The Primary Objective:</u>

Assessment of bacterial translocation by venous blood bacterial DNA in response to diet containing soya lecithin emulsifier, in comparison to control period of low-emulsifier diet. Measured as responding to pre and post dietary treatment as gene copy number per ml (as per Gutiérrez *et al.*, 2014 [6]), but using more validated universal qPCR primer sets (including total bacteria, phyla- and class-specific primers as described by Bacchetti De Gregoris *et al.*, 2011 [7]). These data will be backed up by measurement in venous blood of circulating soluble CD14 and LPS binding protein as additional markers of bacterial translocation [8] – see secondary endpoints.

Secondary endpoints:

Changes in comparison to control (expressed as pre-post change per diet period)

- Faecal calprotectin (Store frozen – ELISA @Campbell Lab, UoL)
- Faecal volatile organics compounds [VOCs] (Store frozen – GC-MS @Probert lab, UoL) [9].
- Faecal microbiota and DNA extraction (Process for SCFA and DNA extraction within 2 weeks, then store frozen – bacterial 16S rRNA gene sequencing @Walker Lab, Rowett; UoA).
- Plasma highly sensitive C-reactive protein [hsCRP] (Store frozen – ELISA @Campbell Lab, UoL; T1,2,3,4 overnight fasted).
- Plasma soluble CD14 and LPS binding protein (Store frozen - ELISA @Campbell Lab, UoL; T1,2,3,4 overnight fasted).
- Plasma fasting blood glucose and up to 3 hours after OGTT (KONE, Rowett; T1,2,3,4 as Time 0, 30, 60, 90, 120 minutes).
- Plasma fasting lipid profile (KONE, Rowett UoA, T1, 2, 3, 4; overnight fasted)
- Plasma fasted insulin profile and up to 2 hours postprandial OGTT (KONE, Rowett UoA; T1,2,3,4 as Time 0, 30, 60, 90, 120 minutes).
  (with calculation of HOMA and Matsuda index as an estimate of insulin sensitivity [10]
- Plasma trimethylamine-N-oxide (TMAO) as a measure of cardiovascular risk [11] (Rowett UoA; T1,2,3,4 as overnight fasted).

3.0 Study design

Diet trial with randomised sequence of delivery of two diets.

Each subject will participate in a 43-day study design as follows:

- 7 days food diary (recording at home, usual diet) (Days -1 to -7)
- 14 days intervention diet (order randomised as low-emulsifier or high-emulsifier fed as low-calorie diet at 100% resting metabolic rate [RMR]) (Days 8-21)
- 7 days washout - food diary (recording at home, usual diet) (Days 22-28)
- 14 days intervention diet (order randomised as low-emulsifier or high-emulsifier fed as low-calorie diet at 100% RMR) (Days 29-42)

- Last test day on Day 43

3.1 Diet & Study Setting

All food is prepared at the Human Nutrition Unit, Rowett Institute and all measurements. Diets are provided as individual diets calculated to provide 100% resting energy requirements. The lecithin supplement will be soya lecithin granules* (Lamberts®, a food grade product, manufactured under GMP conditions) given as 7.5 g twice daily, incorporated into juices.

*Containing, per 7.5 g; – phosphatidyl choline 1.7 g, phosphatidyl ethanolamine 1.5 g, phosphatidyl inositol 1.1 g, phosphatidic acid 0.6 g, phosphatidyl serine 0.075 g and fatty acids 3.8 g of which 0.9 g saturated, 0.3 g monounsaturated, 2.5 g polyunsaturated.

Selection of the source /dose of soy lecithin is based on the following: 7.5 g granules bi-daily (15g daily dose).

(i) 15g/day is substantially greater than the typical dietary intake of up to 5g/day so should be sufficient to test for possible effects in what is a relatively short-term study;

(ii) Based on previous work by Cobb *et al*. [12] gave healthy volunteers 7.5g three times daily for 4 weeks and did not report any negative effects

(iii) The supplier's (Lamberts®) web site (which implies that lecithin is good for you) states, "Daily intake 7.5 to 15 grams". Lamberts® as supplier has been selected because they manufacture in the UK according to the stringent pharmaceutical standards of Good Manufacturing Practice (GMP). The soy lecithin granules have "a pleasant mild taste" and are mixed into a fruit/dairy smoothie matrix for stability and compliance.

3.2 Participant screening

Initially, participants (n=20 to complete, screening will continue until study completion) will attend a screening visit at the Rowett's Human Nutrition Unit (HNU). This will involve

- self-report general health screening questionnaire
- Height and body weight
- Blood pressure
- completing consent paperwork (PIS and consent form)
- contact details to arrange next appointment to attend the Human Nutrition Unit

Volunteers will then complete a Baseline (Day 1) visit involving:

- RMR measurement for preparation of individual diets
- Height and body weight
- Blood pressure
- Waist and Hip circumference
- Body fat% assessed by BodPod

Volunteers will then be given a 7-day weighed intake food diary to be completed during the initial week washout period of the study (no probiotic or prebiotics to be consumed or antibiotics) to establish their regular eating habits and given a faecal pot to provide a baseline stool sample.

3.3 Study Visits

Subsequent study visits will consist of 4 test days when volunteers will be asked to fast overnight before presenting at the HNU. In total there will a baseline visit followed by 4 study visits representing pre-post each diet treatment.

For Test days 1,2,3,4 (Protocol Days 8, 22, 29 and 43; one 6ml EDTA tube and one 6ml LiHep tube and one 2.7ml Li-Hep tube, total 14.7ml whole blood)

- Participants are asked to bring a fresh (<16 h) faecal sample for SCFA and DNA extraction; lab staff to report Bristol stool chart (D8, D22, D29 and D43) & collect VOC.

- Cannulation for blood sampling (D8, D22, D29, D43)
    - T0 Lipid profile (2x200µl plasma), whole blood in LiHep tube 1
    - T0 Glucose (2x200µl plasma), whole blood in LiHep tube 1
    - T0 Insulin (2x200µl plasma), whole blood in EDTA tube 1
    - T0 Trimethylamine-N-oxide (TMAO), (2x200µl plasma), whole blood in LiHep tube 1
    - T0 plasma hsCRP (2.7ml whole blood, (2x100µl plasma), whole blood in EDTA tube 1
    - T0 soluble CD14 and LPS binding protein (2x100µl plasma), whole blood in EDTA tube
    - T0 bacterial DNA in sterile tube (1ml whole blood), whole blood in Li-Hep tube

- After ingestion of 75g glucose (Days 8, 22, 29 and 43; one 2.7ml EDTA tube and one 2.7ml LiHep tube for each timepoint, total 21.6ml whole blood)
    - T30, T60, T90, T120 Glucose (2 x 200µl plasma for each timepoint), whole blood in EDTA tube 2,3,4,5
    - T30, T60, T90, T120 Insulin (2 x 200µl plasma for each timepoint) whole blood in LiHep tube 2,3,4,5
- GI discomfort questionnaire [13] (Storey et al 2007, D8, D22, D29, D43)

Total blood is 36.3ml each test day (equivalent to 2 tablespoons) or 145.2 ml over the entire protocol (less than one blood donation). Subjects will receive reimbursement of travel expenses and a small gratuity (up to £50).

3.4 Timetable

Volunteer schedule for 2019, to complete 20 (10 men and 10 women)

Appointed post-doc to start 4th Feb 2019

2 volunteers – May to June 2019

2 volunteers – June to July 2019

2 volunteers – July to August 2019

4 volunteers – August to September 2019

4 volunteers – September to October 2019

4 volunteers – October to November 2019

2 volunteers – November to December 2019

Analysis – Jan to April 2020

Write up –April to June 2020

3.5 Study Population

Twenty completing participants. Should there be any drop-put, we will continue to replace participants until 20 have completed the trial.

Inclusion Criteria: Men and women adults aged 18+ years; BMI ranging from 27-40 kg/m^2; measured at screening visit.

Exclusion Criteria: Potential volunteers will be asked to fill in a health questionnaire to assess their suitability for the study. This information will allow us to exclude and subjects with:

Medication exclusion criteria - antibiotic use within the past 3 months (due to impact on gut microbiota), statins (current), aspirin (current).

Medical exclusion criteria– chronic inflammatory disorders such as rheumatoid arthritis, inflammatory bowel disease; food allergies; self-reported food sensitivity or intolerance; diagnosis of diabetes; pregnant or breastfeeding; unsuitable veins for blood sampling; inability to speak, read and understand English; unable to comply to alcohol free diet for 5 weeks; consumption of nutrition supplements. Soy allergy or intolerance.

3.6 Participant Selection and Enrolment

Recruitment of volunteers will primarily be through press releases and radio adverts followed by word-of-mouth. We will also contact volunteers from previous studies who have consented specifically that they are willing to take part in human intervention studies. We will be putting up small poster adverts in the local Aberdeen area (in community centres, gyms and shops) as well as the University of Aberdeen advertisement screens.

3.6.1 Consenting Participants

Participants will receive the Participant Information sheet when they first express an interest in the study and will be asked to return a copy of the general information form to the research team to provide their contact details and basic eligibility information. When they attend the HNU for their first visit they will meet a member of the Research Team who is trained in taking informed consent. This person will ensure the subject understands what the study entails and answer any queries they have. If the subject is happy to proceed then

they and the researcher will sign & date two copies of the study Consent Form. The subject will keep one copy of this form and the researcher the other.

3.6.2 Screening for Eligibility

Participants will undertake a screening test day to ensure eligibility. Following the screening all results will be assessed by HNU GP and local PI to ensure eligibility. Participants will be deemed eligible if (i) their health and wellbeing is not at risk by undertaking the study and (ii) they are not on any medication or have any health condition which will impact study outcomes.

The screening will include:

- General health questionnaire
- Height and weight taken to fulfil BMI criteria

Only eligible volunteers based on meeting the inclusion and exclusion criteria will be recruited for this study up to the required number per group. Once this is reached, additional interested volunteers will be thanked for their interest and no data will be collected. Participants who express an interest and attend the health screening will be required to sign the consent form before undertaking any of the screening measures. Participants who are deemed ineligible during the screening will be thanked for their interest and provided with their health screening results. Ineligible participants will be replaced to meet the 20 participants required to complete the study. If participants do not meet the requirements based on any health markers falling outside of normal values -the participant's results will be sent to their GP. Screening results from these participants will be maintained for audit purposes.

4.0 Randomisation and Blinding

Randomization for treatment order will be conducted by computer generation by the statistical team to create a treatment order. A randomisation order will be generated by Dr Graham Horgan (Biomathematics and Statistics Scotland) and participants will be assigned in the order in which they are assigned to the study following their health screening and

confirmation of eligibility. The randomisation uses a mathematical algorithm validated for generation of random sequences.
The study will be conducted in a non-blinded method with diets colour coded.

5.0 Withdrawal Procedures

If any participant wishes to withdraw from the study at any point after recruitment, any data already collected with consent will be retained and used in the study. No further data will be collected, or any other research procedures carried out on or in relation to the participant after they withdraw. A "withdraw from study form" will be completed and attached to the participants record. Unless the participant specifically withdraws consent to use any data already collected, all data to the date of withdrawal will be retained and used for analysis purposes.

6.0 Study and Safety Assessments

The risks associated with participation in this project are minimal. Minor risks are associated with the collection of blood samples.

Blood Collection: Blood collection will be performed by trained and experienced phlebotomists or research nurses. Venepuncture blood collection is not associated with risks greater than that associated with a routine blood test. There may be mild discomfort and a small amount of localised bruising as a result of blood collection and some participants may feel light headed or faint, but no more than is experienced with a routine blood test. Each member of the research team also has extensive experience in blood collection and handling. A phlebotomy checklist will be completed with each blood test to confirm any previous problems with blood tests recent blood donations and current illness to minimise risks.

Any medical values which fall outside of normal ranges will be reported to the HNU unit manager, recorded appropriately and the participant's GP notified (with consent). All measurements will be undertaken by trained research staff in accordance with authorised

SOPS and nursing staff are on site to maintain subject health and safety. All adverse events will also be reported to the HNU unit manager and Sponsor.

7.0 Data Collection and Management

All data will be collected by the research team and bloods collected by the phlebotomists or research nurse. Data will be collected using validated questionnaires, the collection of biological samples (blood, faeces), and measures of human physiology with calibrated research equipment. The research team are trained in the appropriate data collection methods and data collection will be completed according to authorised SOPS. All data will be stored in anonymised form in locked cabinets at the Rowett Institute in the Human Nutrition Unit or on secure shared drives accessible by the research team. Biological samples will be stored in secured 70°C degree freezers as until analysis. Data will be identifiable only by the research team in order to link health outcomes and samples to subjects. All electronic data will be stored "ad infinitum" on the University of Aberdeen central server. 16S rRNA gene–based gut microbiota profiling sequence data would need to be uploaded to a publicly accessible data repository such as the European Nucleotide Archive prior to publication. Only bacterial profiles will be available, and sample will remain anonymised so that these profiles cannot be linked to specific individuals. Archiving will only occur once all relevant paper data has been published. It is expected that paper data will be archived after 5 years following publication of the project; in line with the institutional retention schedule.

8.0 Labs and Samples Analysis

Faecal samples will be used to determine gut microbiota, SCFA, VOCs and calprotectin. Samples will be collected into a pre-lined faecal collection pot when the participant needs to defecate. The participant will be required to seal the container and return it to the institute within 12 hours for effective microbial microbiome and metabolome profiling (e.g. bacterial 16S rRNA gene/SCFAs/VOCs analysis [9, 14, 15]. Calprotectin concentration in faeces is higher than in plasma and significantly increased levels of calprotectin in stool are found in patients with bowel inflammation (e.g. IBD), whereas it is not elevated in patients with non-organic, rather functional diseases like irritable bowel syndrome (IBS) [16].

Blood samples will be collected by a trained phlebotomist or research nurse into 2 separate vacutainer tubes (EDTA and lithium hep tubes). Blood samples will be immediately spun in the centrifuge and plasma aliquots placed into Eppendorf tubes (insulin and TMAO (UoA) and hsCRP, sCD14, LPS binding protein (UoL)) and KONE cups (for glucose and lipid analysis). Plasma samples will be stored in a -70°C degree freezer until analysis.

All samples will be collected and stored in anonymised forms. All freezers are calibrated and monitored by the research team. All laboratory analysis of faecal and blood samples will be undertaken at the Rowett Institute by trained laboratory technicians in the analytical laboratories.

Samples will be stored until the study has been completed and then for further 5 years to allow for potential additional analysis in future ethically reviewed and approved studies. After this period, all samples will be destroyed.

9.0 Statistics and Data Analysis

9.1 Sample Size Calculation

Emulsifier circulating bacterial DNA power calculation - The sample size chosen to give 80% power to exclude 50% increase in circulating bacterial DNA. Human studies to date have shown up to 10-fold increase in circulating bacterial DNA in CVD but a 50% increase, as has been demonstrated for example after an acute alcohol binge, is a more realistic expectation for the proposed human dietary study.

The largest published data set of peripheral venous blood bacterial 16S rRNA gene measurements is that published by Dinakaran *et al.* [17] who report data from 40 healthy controls and 80 patients with cardiovascular disease. Values for healthy controls for 16S rRNA genes were median 411.73 gene copy number per mL, IQR 186.62-617.16 (values for individuals with cardiovascular disease were more than ten times higher). By assuming that the mean was approximately similar to the median and extrapolating an approximate value for standard deviation (34/25x225=306) the power calculation shows that a sample size of 20 will give 80% power at P=0.05 of excluding a 50% increase in circulating bacterial DNA.

Sample size for a paired or single sample Student t test.

Alpha = 0.05; Power = 0.8; Difference of mean from zero = 206; Standard deviation = 306
Estimated minimum sample size = 20 pairs; Degrees of freedom = 19
Allowing for drop out typical of volunteer dietary intervention studies, we would aim to recruit 22 volunteers for the study, to complete 20.

9.2 Proposed Analysis

Data analyses will be reviewed by independent Statistician, Dr Graham Horgan from BioSS, included as a subcontractor. The entire trial management team will be responsible for data analysis.

Missing Data - Data analysis will be performed using linear mixed models which enables the inclusion of all participants within the analysis even with missing time points. When entering data into excel spreadsheets, missing data will be left blank. Blank cells will be checked by statistician Dr Horgan during statistical analysis to confirm the data as uncollected/ erroneous as opposed to incorrectly transcribed.
The loss of data will be minimised by immediate saving of results on test days and storage of data on the University central server which is backed up daily. Consistent reminders to participants of study protocol, adherence to SOPs and calibration of equipment will be used to minimise the chances of errors.

10.0 Study Management and Oversight Arrangements

The trial/study will be co-ordinated by Professor Alexandra Johnstone and Dr Alan Walker and Dr Dominic Partridge. This group will have overall responsibility for the conduct and running of the study and will report to Grant collaborators via Professor Barry Campbell. Regular meetings will be held to ensure consistent communication between all parties of the research team. The PI and all institutions involved in the study shall permit study related monitoring, audits, and review. The PI agrees to allow the Sponsor or, representatives of the Sponsor, direct access to all study records and source documentation if required.

11.0 Good Clinical Practice

Ethical Conduct of the Study - The study will be conducted in compliance with the 'principles of GCP' for non CTIMP/MHRA studies. In addition to Sponsorship approval, a favourable ethical opinion will be obtained from an Ethics Review Board.

12.0 Confidentiality

All laboratory specimens, sample forms, reports, and other records will be anonymised in a manner designed to maintain participant confidentiality. All records will be kept in a secure storage area with limited access to study staff only. The CI and study staff involved with this study will not disclose or use for any purpose other than performance of the study, any data, record, or other unpublished, confidential information disclosed to those individuals for the purpose of the study. Prior written agreement from the Sponsor or its designee will be obtained for the disclosure of any said confidential information to other parties.
Upon participant consent, participant contact details may be kept by the research team to re-contact participants for future ethically approved studies. Contact information will be kept separate to all anonymised research data.

12.1 Data Protection

The local PI and study staff involved with this project will comply with the requirements of the General Data Protection Regulations (GDPR) and the Data Protection Act 2018. Access to collated participant data will be restricted to the CI and appropriate study staff. Computers used to collate the data will have limited access measures via usernames and passwords. Published results will not contain any personal data that could allow identification of individual participants.

12.2 Insurance and Indemnity

The University of Aberdeen is sponsoring the study.
Insurance: The University of Aberdeen will obtain and hold a policy of Public Liability Insurance for legal liabilities arising from the study.
Indemnity: The Sponsor does not provide study participants with indemnity in relation to participation in the Study but has insurance for legal liability as described above.

13.0 Study Conduct Responsibilities

The PI will seek approval for any amendments to the Protocol or other study documents from the Sponsor and REC. Amendments to the protocol or other study documents will not be implemented without these approvals.

13.1 Study Record Retention

Archiving of paper study documents will be conducted in line the University of Aberdeen's archival policy and will be stored for 05 years after the end date of the study. An electronic copy of the data will be held indefinitely by the PI.
End of Study - The end of study visit for volunteers is defined as the last visit at study day 43 with the consent to contact participants again with their feedback report.

14.0 Reporting, Publication and Notification of Results

Authorship Policy - Ownership of the data arising from this study resides with the study team and their respective employers and the funder MRC. On completion of the study, the study data will be analysed and tabulated, and a study report will be prepared.

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