STUDY PROTOCOL

Title: Probiotic beer to enhance gut health and immune system function.

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PRINCIPAL INVESTIGATOR:

1. Khoo Chin Meng, Senior Consultant Endocrinologist, National University Hospital, Assistant Professor, Yong Loo Lin School of Medicine, National University of Singapore

CO-INVESTIGATORS:

1. Liu Shao Quan, Associate Professor, Department of Food Science & Technology, National University of Singapore
2. Liu Mei Hui, Senior Lecturer, Department of Food Science & Technology, National University of Singapore

STUDY SITE:

1. Investigational Medicine Unit, National University Hospital, Yong Loo Lin School of Medicine, National University of Singapore

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1. BACKGROUND

1.1 Introduction

Inflammation in the body is increasingly being identified as one of the factors that contribute to the exacerbation or the onset of diseases, especially in regards to today's context of metabolic diseases like cardiovascular diseases and type II diabetes. Inflammation occurs as part of the body's normal response to damaged surrounding tissues and cells which, if persistent, may turn chronic and result in illness (Weiss, 2008). Inflammation is usually tackled by the immune system, where the various types of white blood cells and other immune cells will deliver signals to fight and alleviate the damage.

Beer is an alcohol-containing beverage that is one of the culturally oldest beverages in the world (Preedy, 2009). It is considered a social drink that is widely consumed. While the excessive consumption of alcohol is widely known to be correlated negatively with health, there have been increasing evidences showing that moderate alcohol consumption has a beneficial impact on health, in particular towards immunity and research has proven that it is a potent immunomodulator (Szabo & Mandrekar, 2009).

The emerging field of microbiome has also shown that probiotics have a positive effect on gut health. Probiotics exhibit a therapeutic effect on immune responses, and even more so since 70% of our immune system lies in our gut (Vighi et al., 2008). Probiotics supplementation has also been identified as one of the ways to support our gut health.

These immune responses that the moderate consumption of alcohol and probiotics each demonstrated are geared towards an anti-inflammatory immune response which is associated with a better prognosis of inflammation in the body (Romeo et al., 2007). The combination of a probiotic strain with moderate beer consumption is thus expected/hypothesized to improve immunological outcomes towards an enhanced anti-inflammatory effect and enhanced gut health.

2. STUDY RATIONALE

2.1. Rationale for Study

Studies have shown that moderate alcohol consumption and probiotics have each shown immunomodulatory anti-inflammatory effects. However, to our knowledge, the effect of adding a probiotic strain to a beer drink, together with moderate alcohol consumption, on immunity and gut microbiome has yet to be studied. The probiotic beer used in this study taps on this unexplored research area and may potentially serve as a more healthful option to consumers than normal beer in the future, given the vast popularity of this beverage and probiotics.

2.2. Rationale for Test Foods

The beers used in this study will be normal beer and probiotic beer. Each beer can will have

3.5-5% alcohol, with a total volume of 330mL per can. The ingredients used in the beer are water, grains (Barke Pilsner, Floor-Malted Bohemian Wheat, Golden Naked Oats, and dextrin malt), raspberry puree, and yeast. Lactic acid bacteria (*Lactobacillus paracasei* Lpc-37®, or *Lactobacillus paracasei* LAFTI®L26) will be added for the probiotic beer.

Beer production took place at Brewerkz, a licensed manufacturer. The milled grains were mashed with brewing water and subsequently boiled to produce wort (unhopped, wheat-based). The wort was then inoculated with a probiotic strain and *Saccharomyces cerevisiae* yeast (SafAle S-04). After fermentation and cold crash, the yeast accumulated at the bottom of the fermentation tank was removed. The beer was then force-carbonated, packaged (in cans or kegs), and kept cold during storage. Probiotic cell count was determined to ensure that the final beer contains at least 1 billion colony-forming units (CFU) per serving at the time of consumption.

2.3. Rationale for Study Population

This study will recruit 30 Chinese healthy male participants. The outcome of interest is the change in immunological parameters to observe an improvement in anti-inflammatory effect, as well as any changes in gut microbiota.

2.4. Rationale for Study Design

A within-subject crossover study design is utilised to compare the immunological outcomes of moderate beer drinking and moderate probiotic beer consumption.

3. HYPOTHESIS AND OBJECTIVE

3.1. Hypothesis

The moderate consumption of probiotic beer is hypothesised to improve gut health and immunological parameters.

3.2. Objective

The objective of this research is to study the immunological and gut microbiome effects of moderate probiotic beer consumption.

4. POTENTIAL RISKS AND BENEFITS

4.1. Potential Risks

1. Minimal risk is anticipated. Some discomfort, pain, bleeding or bruising at the site of the needle stick may occur during collection of blood samples. There is a risk of vein collapse during the venepuncture procedure. In the case of such events, blood will be attempted to be drawn from a different vein.

2. The beers used in this study will be brewed and packaged by a licensed production company, Brewerkz. The ingredients of the beers will be made known to the subject before their participation in the study. In a rare unfortunate event, a food allergy is possible. In the case of a food allergy, the subject will be removed from the study and directed to receive proper medical treatment. Brewerkz is committed to ensure that there is no contamination in the beer production. All equipment used in the manufacturing of the beer undergoes cleaning with strong cleaning solution at high temperature and further sanitized with SFA approved acid solution (peroxyacetic acid, 8th schedule). The effectiveness of the cleaning and sanitation process is confirmed using an onsite test kit (ATP Luminometry) as an indicator for presence or absence of contaminants and cleanliness level. Before piping/ connectors are attached to inlet or outlet ports of tanks, the attachment surfaces are rinsed with sanitizer solution (Isopropyl alcohol) and immediately burned to disinfect the area.

4.2. Potential Benefits

Results from the study will be meaningful to the development of a more healthful alternative to normal beer. There will be no direct benefit to the subjects.

5. STUDY POPULATION

5.1. Sample size

The sample size for the present study was calculated with 90% power and a two-sided 0.05 significance level. At least 21 subjects would be needed for this study to find a significant increase in cytokine levels. Based on the 2007 study by Romeo et al. titled 'Changes in the Immune System after Moderate Beer Consumption', there was a significant increase in the cytokine IFNg in men comparing between a 30-day abstinence period and a 30-day moderate beer consumption period. From their data, the standard deviation of the difference between the two periods and the difference in their means was derived as 8287 pg/ml and 6208 pg/ml respectively. Using these values and a 90% power with 0.05 significance level, the minimum sample size was calculated to be 21. To account for a loss to follow-up, 30 subjects will be recruited for this study.

5.2. Recruitment procedure

Recruitment of subjects will be made through advertisement (i.e. posters) and word of mouth. Potential subjects will be contacted and screened for eligibility according to the inclusion and exclusion criteria by the research personnel. The research personnel will identify eligible candidates and will explain the study rationale, procedures, and potential risks and benefits as stated in the study protocol. The Participant Information Sheet (PIS) will be provided for the candidates to read prior to their decision to participate in the study. The candidates will be encouraged to ask questions about the study and have adequate time to make a decision. Informed consent will be acquired from eligible candidates before enrolment into the study. Subsequent visits and any other administrative details will be arranged between the research personnel and participants.

5.3. Inclusion criteria

Subjects must meet all of the inclusion criteria to participate in this study:

1. Ability to give informed consent.
2. 21 – 60 years of age (inclusive) at screening.
3. Healthy male, as determined by medical history, physical examination and laboratory results within normal reference range for the population or investigator site, or results with acceptable deviations that are judged to be not clinically significant by the investigator.
4. Race must be Chinese.
5. Willing to consume 1 beer can per day for 14 days.
6. Not on any regular medications (western/ traditional).
7. No family history of alcoholism.

5.4. Exclusion criteria

1. Female.
2. A current smoker, have smoked, or is a user of tobacco products for the past 2 years.
3. History or presence of current lipid and cardiovascular disorders, respiratory, hepatic, renal, gastrointestinal, endocrine, lipid disorder, haematological, malignancy or neurological disorders capable of significantly altering the performance of the biomarker panel; or of interfering with the interpretation of data.
4. History of alcoholism, alcohol dependence, alcohol abuse, alcohol allergy and/or any other alcohol use disorders.
5. History of Type 1/ Type 2 diabetes and use of anti-diabetic medications in the past.
6. Regular use of medication that are known to have an effect on immune function.
7. Regular use of aspirin.
8. A naïve alcohol drinker.
9. Persons with known or ongoing psychiatric disorders or drug abuse within 3 years.
10. Active infection requiring systemic antiviral or antimicrobial therapy that will not be completed prior to first visit of the study.
11. Treatment with any investigational drug, or biological agent within one (1) month of screening or plans to enter into an investigational drug/ biological agent study during the duration of this study.
12. Significant change in weight (+/- 5%) during the past month.
13. Antibiotic use in the past 2 months.

5.5. Withdrawal criteria

Enrolled subjects may withdraw from the study:

1. In the case of any serious adverse event.

2. If they decide to do so, at any time, and irrespective of the reason. Reasons provided by the subjects will be documented.

3. If study site participation is discontinued, when the investigator judges it necessary for medical, safety, regulatory or other reasons consistent with applicable laws, regulations and good clinical practice.

6. STUDY DESIGN

6.1. Study method

The experimental design used in this study is a randomised, controlled, within-subject cross-over design. Each individual will undergo a 2-week intervention each of moderate beer consumption and moderate probiotic beer consumption, with a 1-week period of washout in between. The total study duration will be 5 weeks. According to the Health Promotion Board (HPB), the recommendation for safe alcohol consumption is two standard drinks a day for men. For beer, one standard drink is defined as one can of beer at 330ml. The beer used in this study is prepared at 330ml in one can (3.5-5% alcohol) in compliance with one standard beer drink. Only males will be recruited in this study, and they are to consume only one can of normal beer or probiotic beer per day during the study periods, which is within the safe moderate alcohol consumption standards set by HPB.

6.2. Randomisation

Randomization will be done using the research randomizer tool at http://randomizer.org. 30 sets will be generated, with each set being randomly assigned a sequence of either (1),(2) or (2),(1). (1) represents the study arm with probiotic beer, while (2) represents the study arm with normal beer.

6.3. Run-in period

Before each study visit, participants will be informed by the research personnel on the following study requirements:

1. Fast for 10-12 hours prior to reporting for the study visit.
2. Avoid foods high in fat (e.g. fried food) for 24 hours prior to reporting for the study visit.
3. Avoid caffeinated beverages 4-6 hours before reporting for the study visit.
4. Avoid strenuous exercises 24 hours prior to reporting for the study visit.

Subjects are also required to abstain from alcohol and probiotic-containing foods, drinks, or supplements for one week prior to the commencement of the study and during the course of

the study itself. No other alcohol and probiotic-containing foods should be consumed apart from the beer or probiotic beer provided during this duration.

6.4. Study visits

- **Visit 1: Screening**

Individuals will be scheduled to attend a screening session. Research personnel will guide the individual through the inclusion and exclusion criteria to validate their eligibility for the study. Upon confirming that the subject is an eligible study subject, research personnel will explain the study in detail to them in the presence of a witness, and they will be given time to read the approved copy of the informed consent form and the opportunity to ask any questions. Following which, a written informed consent from the individual will be obtained and they will keep a copy of the form.

Anthropometric measurements will be collected, including: height, weight, waist and hip circumference, blood pressure, and body composition measurement using a body composition analyser. A total of 22mL of blood will also be drawn during this visit to test for baseline biomarkers, such as renal and liver function, lipid profile, HbA1c, and blood glucose levels. Plasma will also be obtained and stored to be analysed for further genotyping/biochemical markers. Stool samples will also be collected via a spatula and collection kit for gut microbiota profiling using stool sample collection kits (OMR-200, DNA Genotek, Ontario, Canada) that will be issued during this visit. Subjects are to bring back the stool samples on their next visit. The research personnel will then proceed to schedule the next study visit for the subject.

Subjects are also required to start abstaining from alcohol and probiotic-containing foods, drinks, or supplements for one week prior to the commencement of the study (i.e. Visit 2) and during the course of the study itself. No other alcohol and probiotic-containing foods should be consumed apart from the beer or probiotic beer provided during this duration.

- **Visit 2: Pre-intervention blood collection (first arm)**

This visit marks the start of the first study arm. A total of 34mL of blood will be drawn during this visit from the enrolled subjects to test for baseline measurements and for biomarkers including C-reactive protein, white blood cell count, and cytokine panels. Plasma will be also obtained and stored to be analysed for further genotyping/biochemical markers. Stool samples will also be collected from the stool collection kit issued during the screening visit. Another stool collection kit will also be issued for the subjects to bring back their stool samples on their next visit. Next, subjects will be supplied with either normal beer or probiotic beer cans (3.5-5% alcohol, 330ml each), to be consumed only when they have no other dexterity tasks to perform (after working hours, after driving, etc) at the end of the day for the next 14 days. Subjects are to consume only one beer can per day.

- **Visit 3: Post-intervention blood collection (first arm)**

This visit marks the end of the first study arm. A total of 34mL of blood will be drawn during this visit from the subjects to test for baseline measurements and for biomarkers including C-reactive protein, white blood cell count, and cytokine panels again after 14 days of intervention.

Plasma will also be obtained and stored to be analysed for further genotyping/biochemical markers. Stool samples will also be collected from the stool collection kit issued during the previous visit. Another stool collection kit will also be issued for the subjects to bring back their stool samples on their next visit. Subjects will then undergo a 1-week washout period before they enter the next arm.

- **Visit 4: Pre-intervention blood collection (second arm)**

This visit marks the start of the next study arm. A total of 34mL of blood will be drawn during this visit from the subjects to test for baseline measurements and for biomarkers including C-reactive protein, white blood cell count, and cytokine panels. Plasma will be also obtained and stored to be analysed for further genotyping/biochemical markers. Stool samples will also be collected from the stool collection kit issued during the previous visit. A final stool collection kit will also be issued for the subjects to bring back their stool samples on their next visit. Subjects will then be supplied with either normal beer or probiotic beer cans (3.5-5% alcohol, 330ml each), depending on which arm they started with first, to be consumed only when they have no other dexterity tasks to perform (after working hours, after driving, etc) at the end of the day for the next 14 days. Subjects are to consume only one beer can per day.

- **Visit 5: Post-intervention blood collection (second arm)**

This visit marks the end of the second study arm. A total of 34mL of blood will be drawn during this visit from the subjects to test for baseline measurements and for biomarkers including C-reactive protein, white blood cell count, and cytokine panels. Plasma will be also obtained and stored to be analysed for further genotyping/biochemical markers. Stool samples will also be collected from the stool collection kit issued during the previous visit.

There will be blood collection during all visits. In total, approximately 158mL (=10 to 11 tablespoons) of blood will be collected for this study. For comparison, about 450 mL (=30 tablespoons) of blood is the amount taken when a person donates blood. Any leftover blood samples will be stored in a -80°C freezer for no longer than 5 years after study completion and will be used for future general research only if subjects have given informed consent.

6.5. Incidental Finding Management Plan

6.5.1. Description of the Incidental Findings (IF) that may be returned

IFs that may be returned are blood test results that are outside the expected normal range. Blood test results in this study includes liver panel, renal panel, lipid panel, insulin, glucose (fasting), HbA1c, C-reactive protein, and full blood count.

6.5.2. Plans to verify the presence of a suspected IF

In the informed consent form, participants will be asked their wish to re-identify and be notified in the case of an incidental finding. The study team will keep a record of answer (Yes or No) in a study log to keep track. Blood test results will be reviewed by a research assistant and

any test results not within range will be noted and communicated to the PI to determine if the result is clinically significant and actionable.

6.5.3. The mode of communication to be used and the limits of effort of re-contacting

The mode of communication used to notify the participant will be face-to-face or via phone call. The participant will be notified face-to-face by the last visit, if the IF was found before the participant's last visit.

If the IF was found after the last visit, the findings will be communicated via phone call. The limits for this effort of re-contacting will be that the participant cannot be reached via phone. In such cases, the research team will attempt to call the participant again at two separate timings. If the participant still cannot be reached, the research team will contact the participant's next-of-kin, whose contact information is stated in the informed consent form by the participant.

6.5.4. Plans for counseling or referrals for the research participant, if applicable

In the event that the findings are determined to be clinically significant and actionable, and if the participant agrees to be re-identified and notified, a study doctor will explain the incidental finding (IF) and will refer the research participant to go for standard-of-care testing and interpretation of results by a qualified professional at an accredited clinical facility.

6.5.5. Any other relevant considerations (e.g. incidental finding has public health implications, participants are less than 21 years old, participants' preference to be re-identified is unknown or subjects are uncontactable etc.)

In the event of the discovery of an IF with public health implications, the research team will notify participants of the IF, regardless of their initial indication in the informed consent form. As only participants aged 21 and above will be recruited for this study, the participant themselves will personally indicate their preference in the consent form. During informed consent, the study coordinator administering the consent will review the consent form and check that the participant has ticked their preference to be re-identified and notified of an IF (Yes or No) in the consent form. This is to ensure that the participant's preference to be re-identified or not is known to the research team. If they agree to be re-identified and notified of an IF (i.e. ticked 'Yes'), they will also need to fill in the contact details of the next-of-kin, as reflected in the form.

7. STATISTICAL METHODS

We will utilise data from blood draws to find the change in C-reactive protein, white blood cell count, and cytokine panels from baseline after each study arm to derive summary statistics for each patient.

Data will be analysed using statistical software (eg. SPSS/Stata), with significance defined as *p*-value of less than 0.05 on a two-sided paired t-test. Other variables will be presented as

mean ± SE. Differences between groups will be analysed using a mixed effect model which can also manage missing data and the presence of unforeseen carryover effects.

For any secondary measurements, paired-t test will also be used to assess baseline versus post-intervention outcomes, compared between the 2 intervention arms.

8. SAFETY MEASUREMENTS

8.1. Definitions

8.1.1. Serious adverse event

A *'serious adverse event'* refers to any event that:

- Results in death
- Is life-threatening
- Requires hospitalization or prolongs existing hospitalization
- Results in a congenital anomaly
- Causes permanent disability or requires medical or surgical intervention to prevent permanent disability
- An important medical event that, based upon appropriate medical judgment, requires medical or surgical intervention to prevent one of the outcomes listed above.

8.1.2. Non-serious adverse event

'Non-serious adverse event' refers to any undesirable symptom or occurrence a subject experiences during participation in a clinical study that does not meet the "serious" criteria listed above.

8.1.3. Anticipated event

'Anticipated event' refers to any event attributed to the underlying condition of the patient being studied, or event attributed to the patient population being studied, or an adverse event anticipated on the basis of prior experience with the procedure and medication.

8.1.4. Unanticipated event

'Unanticipated event' refers to any unanticipated event that cannot be attributed to the underlying condition of the subject being studied or to the subject population, expected events whose frequency or severity exceeds what was anticipated, an event that cannot be attributed to a co-morbid condition or concomitant medication, or an adverse event that was not anticipated on the basis of prior experience with the procedures or drugs used in the study.

8.1.5. Related or possibly related event

'Related event' refers to any event directly or indirectly attributed to the procedure, medication and/or study participation, or events occurring with sufficient frequency to suggest that they are not random.

'Possibly related event' refers to any event with a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research.

8.1.6. Unrelated event

'Unrelated event' refers to any event that would occur regardless of study participation or events that are clearly random occurrences. If the frequency of the event suggests a possible connection to the study procedure, then it should be considered related.

8.2. Collecting, recording and reporting of "Unanticipated Problems Involving Risk to Subjects or Others" – UPIRTSO events to the National Health Group (NHG) Domain Specific Review Boards (DSRB)

UPIRTSO events refers to any incident, experience, or outcome, including adverse events, that meets ALL of the following criteria:

1. **Unexpected**
   Any event occurred that is not consistent with the research procedures as described in the protocol-related documents (i.e., DSRB approved research protocol and informed consent document) or with the characteristics of the subject population being studied in terms of nature, severity and frequency.

2. **Related or possibly related to participation in the research**

   Related, meaning any event directly or indirectly attributed to the procedure, medication and/or study participation, or events occurring with sufficient frequency to suggest that they are not random;

   Possibly related, meaning any event with a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research.

3. **Risk of harm**

   The research places participants or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.

<u>Reporting Timeline of UPIRTSO events to NHG DSRB:</u>

1. Urgent Reporting: All problems involving local deaths, whether related or not, should be reported immediately, within 24 hours after first knowledge by the NHG investigator.

2. Expedited Reporting: All other problems must be reported as soon as possible but not later than 7 calendar days after first knowledge by the NHG investigator.

8.3. Collecting, recording and reporting of Expected Serious Adverse Events (SAEs) to the National Healthcare Group (NHG) Domain Specific Review Boards (DSRB) and Research Institution(s) (RI)

For reporting of **Expected SAEs**, ALL of the following have to be met:

1. **Serious adverse event**

A *'serious adverse event'* refers to any event that:

- Results in death
- Is life-threatening
- Requires hospitalization or prolongs existing hospitalization
- Results in a congenital anomaly
- Causes permanent disability or requires medical or surgical intervention to prevent permanent disability
- An important medical event that, based upon appropriate medical judgment, requires medical or surgical intervention to prevent one of the outcomes listed above.

2. **Expected event**

Any risks or events reported that is listed in in the protocol-related documents (i.e., DSRB approved research protocol and informed consent document).

3. **Related or possibly related to participation in the research**

Related, meaning any event directly or indirectly attributed to the procedure, medication and/or study participation, or events occurring with sufficient frequency to suggest that they are not random;

Possibly related, meaning any event with a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research.

<u>Reporting Timeline of Expected SAEs to NHG DSRB:</u>

All Expected SAEs should be reported as soon as possible but no later than 7 calendar days after first knowledge by the investigator. Any additional relevant information about the events should be reported within 8 calendar days of making the initial report.

8.4. Safety monitoring plan

Safety monitoring will be reviewed daily at every visit for any adverse events throughout the study period. Data integrity will be monitored monthly. The PI or study team member will review the data periodically every 3-6 months.

8.5. Advise on Safe Drinking

Subjects will still be advised on the safe consumption of alcohol though the amount of alcohol to be consumed in the beers is small (3.5-5% alcohol, 330ml per can). Emphasis will be placed

on the consumption of the beers only after there are no other dexterity tasks (after working hours, after driving, etc) to be performed at the end of the day. Subjects will also be strictly cautioned against drink driving.

8.6. Complaint handling

Complaints will be handled initially by research personnel and PI. If the complaint is medical in nature or if it cannot be resolved at that level, a member of the study team will take over. All data will remain confidential and may still be used in the final analysis of the trial.

9. DATA

9.1. Data Entry and Storage

Data will be stored both on paper and electronically. Hardcopy data will be kept under lock and key at the co-PIs' work-station located in the co-PIs' office with access code required for entry. Electronic data will be stored in a standalone-unshared personal computer belonging to the PIs located in the same place with access code required for entry. Only the PI will have immediate access to the research data with sharing of data only to the co-investigators and research assistants. The PI or study team member will review the data periodically every 3-6 months.

At least two study coordinators will be present during the data collection. Study coordinator 1 will measure the data, while study coordinator 2 will check if the data written on the Data Collection Sheet correlates to the data obtained from the measurement. Study coordinator 2 will have to sign on the DCS for each visit to check that the data written is correlated with the measurement, and ensure that this protocol is followed throughout the study. We have included this part in the newly uploaded Version 2 of the DCS. The instruments used to measure anthropometric measurements will also be calibrated, which will ensure complete, authentic, and accurate results.

10. ETHICAL CONSIDERATIONS

10.1. Informed consent

Informed consent will be taken from eligible subjects during the screening visit using the NHG DSRB approved version of the Participation Information Sheet (PIS) and Informed Consent Form (ICF). SGGCP guidelines and ethical principles from the Declaration of Helsinki will be strictly complied to by the research personnel. The research personnel who conducted the informed consent discussion will personally sign and date the ICF.

10.2. Confidentiality of data and patient records

Hardcopy data will be kept under lock and key at the co-PIs' work-station located in the co-PIs' office with access code required for entry. Electronic data will be stored in a standalone-unshared password protected PC belonging to the PI located in the same place. There will be scheduled changes to passwords. There will be encryption of all email or protection by password of all data with patient identifiable information. Only the PIs will have immediate access to the research data with sharing of data available only to the co-investigators on an "as needed" basis. Biological samples will remain coded and stored with the laboratories.

11. RETENTION OF TRIAL DOCUMENTS

Records for all participants, including CRFs, all source documentation (containing evidence to study eligibility, history and physical findings, laboratory data, results of consultations, etc.) as well as IRB records and other regulatory documentation will be retained by the PI in a secure storage facility. The records should be accessible for inspection and copying by authorized authorities. These records will be stored at the PI's workstation under lock and key that is located in the PI's office with access code required for entry.

12. REFERENCES

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