PROTOCOL AND ETHICS DOCUMENT

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The effects of a combined exercise intervention on gut microbiomes and systemic inflammatory biomarkers in NAFLD patients

Understanding the mechanisms to treat NAFLD with exercise

Christiaan D. Hattingh, David Jenkins, Mia A. Schaumberg, James O'Beirne

Contents

1. Title

The effects of a combined exercise intervention on gut microbiomes and systemic inflammatory biomarkers in NAFLD patients.

2. Project Team Roles and Responsibilities

Chief investigator Prof David Jenkins PhD, MSc, BA (Hons)	Dr Jenkins is an exercise physiologist and Professor of Sport and Exercise Science at UniSC. He will provide input on experimental design, interpretation of the results, and manuscript writing.
Co-supervisor Dr Mia Schaumberg PhD, GCHEd, AES ESSAM, BExSS (Hons)	Dr Schaumberg is currently a University of Sunshine Coast (UniSC) Senior Lecturer in Physiology. She leads a growing research group focused on exercise and lifestyle interventions for healthy ageing and dementia prevention. Her investigations extend from laboratory studies to clinical trials and community implementation studies with a translational focus. She will provide input into research design, physical function assessment, and overarching senior research advice.
Nominated Co-supervisor Professor James O'Beirne MBBS(Hons) FRACP FRCP MD EDIC	Professor O'Beirne is a Consultant Hepatologist with a proven track record in clinical investigation and trials in liver disease. He will provide insights into clinical trial design, patient selection and hepatological aspects of the studies.
Student Investigator Mr Christiaan Hattingh BClincExPhys, BBiomedSc(Hons), PhD candidate	Mr Hattingh is a qualified Clinical exercise physiologist with five years of industry experience. He recently completed a Bachelor of biomedical science (Hons) and is currently enrolled as a PhD candidate. He is interested in

	research and academia in tertiary institutions with a particular passion for pathophysiology.
Co-Investigator Dr Marloes Dekker Nitert, PhD, SFHEA	Dr Nitert is an experienced microbiologist from the University of Queensland and will be assisting in gut microbiome analyses.
Research Student/s Honours, Masters, or PhD	Throughout the study duration, suitable students completing research at UniSC in relevant fields (e.g. Exercise Physiology, Biomedical Science, Medical Science) may contribute to the research project. Before undertaking any research activities, such as data collection, each new research team member will be trained by qualified personnel on all standard operating procedures.

3. Site Location

Sports Tower (T1.09)
UniSC Sunshine Coast
90 Sippy Downs Drive Sippy Downs

UniSC Gym
UniSC Sunshine Coast
90 Sippy Downs Drive Sippy Downs

Sunshine Coast Health Institute (SCHI)
Sunshine Coast University Hospital (SCUH)
6 Doherty St, Birtinya QLD 4575

University of Queensland (UQ)
St Lucia QLD 4072

4. Resources

4.1. Rationale/Justification

The purpose of this study is to

i) Examine whether gut microbiome composition, key inflammatory markers, body composition, cardiovascular fitness, and muscular strength are related to the severity of NAFLD and its quality of life through a cross-sectional study.

ii) Examine the influence of a supervised 12-week exercise intervention on changes in relationships between gut microbiomes and inflammatory markers and elucidate possible underlying mechanisms to treat NAFLD.

iii) Inform the development of evidence-based recommendations for diagnostic, exercise, and treatment-related outcomes in patients with NAFLD.

4.2. Funding

Funding for this project will be sourced through the PhD funds of Mr Christiaan Hattingh and consultancy funds of Dr James O'Beirne.

4.3. Project Resources

Project resources, including facilities for exercise testing equipment, DXA scanning, blood collection and analysis, are available at UniSC. All necessary equipment is located on level one of the Sports Tower (T1.09), USC, Sippy Downs and at the Sunshine Coast University Hospital (SCUH) and Health Institute (SCHI).

5. Background

5.1. Literature Review

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent liver disease worldwide (up to a third in some countries)(1–4). It significantly reduces the quality of life (QoL) and increases the severity of comorbidities and other health implications for individuals with the disease (1–8). In addition, the prevalence combined with increased comorbidity risk and severity poses a significant economic burden to society (1–8). Currently, no pharmacological treatment is in place to treat NAFLD directly; therefore, contributing to the most current research to provide a low-cost treatment strategy, both effective and non-invasive, could hold the key to ameliorating and potentially reversing liver disease (1,3,4,9–12).

The main roles of the liver are to process nutrients, toxins other present chemicals in the blood (13). The health and function of the liver are influenced by systemic inflammation, diet, exercise, and body composition; each of these factors is believed to contribute to NAFLD disease risk and progression (14–16). NAFLD is a clinical condition characterised by the presence of steatosis in >5% hepatocytes and lobular inflammation (2,3). NAFLD is due to the lifestyle factors mentioned above in the absence of significant alcohol consumption (2,3). The lipid deposits (steatosis) within the liver impact this vital organ's function and related mechanisms by promoting inflammation and fibrogenesis (3,17,18). NAFLD is also closely associated with metabolic syndrome and shares similar features but is still

distinct (3). The main cause of NAFLD development relates to the patient's lifestyle, which includes diet and physical activity levels (4,11). Obesity is one of the leading factors in the risk of NAFLD occurring due to dyslipidaemia (4,19). This coincides with insulin resistance, as the liver has an important role in glucose homeostasis through gluconeogenesis and glycogenolysis (3,18). NAFLD can start from simple steatosis and progress to cirrhosis and liver cancer (3,13,18,20,21). High-calorie foods, predominantly sugar, combined with a sedentary lifestyle greatly increase the risk of NAFLD development. Other factors contributing to NAFLD development include genetic and metabolic predisposition, with it being known as a multifactorial disease with epigenetic and environmental factors (3,11).

Understanding NAFLD and the methods used for diagnosis are developing gradually. However, much is still unknown about how the other underlying mechanisms contribute to disease pathogenesis. Specific gut microbiomes play a significant role in liver health and function through mechanisms linked by the portal vein (14,16). A high abundance of Proteobacteria in NALFD patients negatively influences systemic and, therefore, liver inflammation through lipopolysaccharides in the outer membrane of the gut via the portal vein (14). Additionally, gut dysbiosis of Bacteroidetes, amongst other phyla of gut microbiomes, impacts energy uptake, insulin resistance and the metabolism of bile acids and choline – all of which impact liver health (14). Increased gut permeability, and production of short-chain fatty acids and lipopolysaccharides via bacterial fermentation, are other factors directly linked with liver steatosis as shown in Figure 1 (14). Exercise has been shown to improve these factors and decrease gut dysbiosis, limiting the negative effects of lipopolysaccharides and increasing the benefits of short-chain fatty acids reaching the liver (14). However, the mechanisms behind the interaction of exercise, in relation to gut microbiomes and the impact on liver disease remain unclear. Recent research has investigated gut microbiomes in other clinical populations such as those diagnosed with cancer or coronary heart disease. Given that the gut microbiome is emerging as having a significant influence on many different markers of health in other clinical populations, examining whether exercise-mediated changes in the gut microbiome may be related to improvements in the health of NAFLD patients warrants attention. Research on the mechanisms of the gut microbiome in NAFLD is in its infancy, with only rat and mouse studies having looked at changes in response to exercise (14,15,22); no human research-controlled trials have been conducted. Exercise has been shown to improve athletes' composition and abundance of gut microbiomes and is associated with lowered inflammatory markers (23–25). Studies of exercise with at least 12 weeks duration, equal to or greater than three days frequency, moderate intensity, and a total of at least 150min/week have shown improvements in steatosis, body weight, serum ferritin, triglycerides, AST and LDL (1,3,4,12).

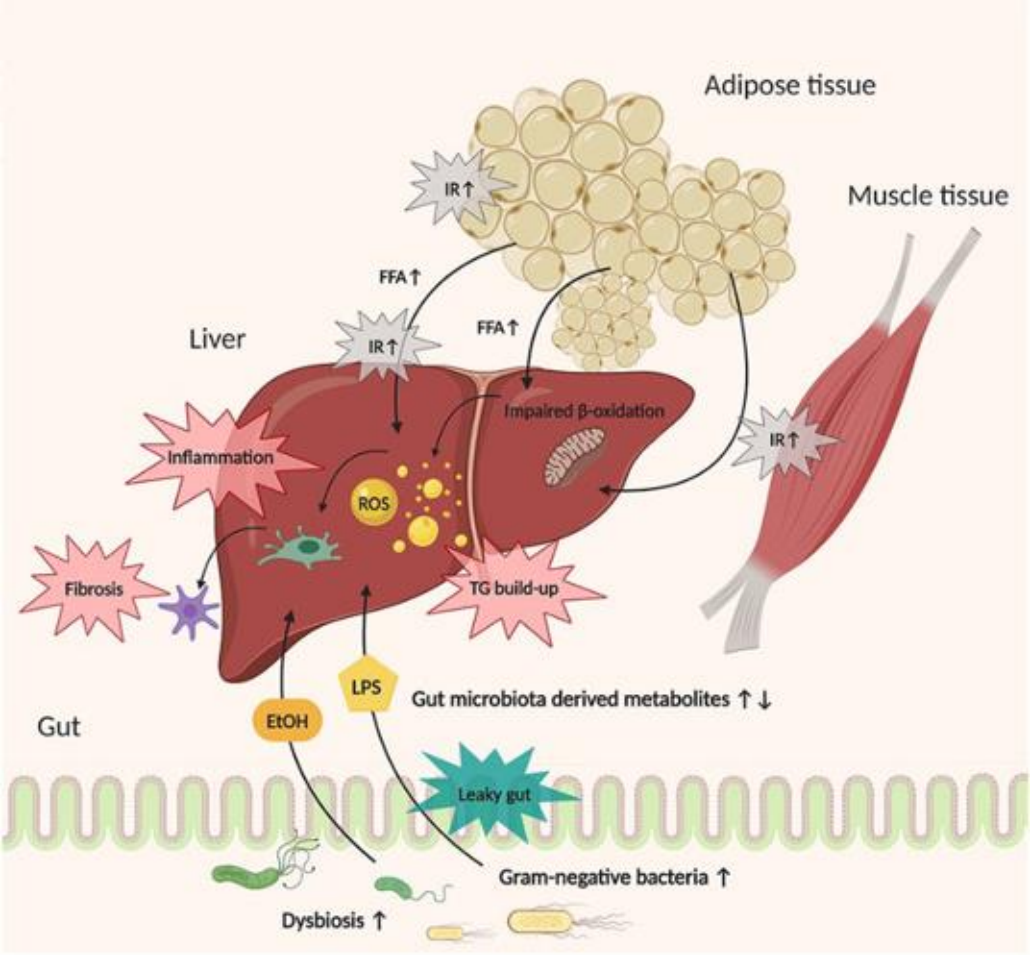

Figure 1: A sedentary lifestyle and unhealthy diet lead to increased triglyceride build-up within the liver. This, along with insulin resistance in peripheral organs and the liver, triggers excessive production of reactive oxygen (ROS) species. Disproportionate ROS activates systemic inflammation and ultimately liver fibrosis. Dysbiosis and increased gut permeability allow lipopolysaccharides and ethanol compounds from gut microbiomes to reach the liver through the portal vein. This, in turn, drives lipid accumulation and inflammation in the liver *(14)*.

Gut microbiome alpha diversity and composition through faecal samples have shown to be different in NAFLD patients compared to a healthy population (14–16). The influence of gut dysbiosis may provide a potential link with NAFLD severity based on current evidence (14–16). However, baseline gut microbiome diversity and composition are heterogenous between NAFLD patients, and there is no current evidence of a specific microbiome phenotype to diagnose NAFLD, this may be due to studies including patients with different stages of NAFLD (14,15). In addition, every person has a unique gut microbiome makeup regardless of disease. Therefore, it can be hard to pinpoint what exact levels these microbiomes should be at to determine disease risk, disease progression, and the best window of opportunity for NAFLD intervention. This study will address the heterogeneity of gut microbiomes through phenotyping the patients during the cohort stage, with each participant acting as their own control during the longitudinal exercise intervention. The link between exercise and improved gut health has also been studied with approving results. Inflammation is also a significant contributing

factor to NAFLD risk, and disease progression with exercise has also been shown to improve this contributing factor and, in turn, affect NAFLD (3,13,17,21).

Central to the proposed study are the outcome measures with liver stiffness measurement (LSM), controlled attenuation parameter (CAP) (markers of liver fibrosis and steatosis), anthropometric measures, gut microbiomes, inflammatory markers, liver blood markers, and general biochemistry markers relevant to identifying the diagnosis of severity of NAFLD. FibroScan, among other clinical technology, determines NAFLD diagnosis and severity (20,26,27). Liver biopsy is the gold standard but is invasive and risky and have been largely replaced by ultrasound techniques such as Fibroscan which has been extensively validated against liver biopsy. (20,26,27). Multiple gut microbiome sampling techniques are used depending on the desired outcome measures, with faecal sample techniques validated for gut microbiome composition and diversity (15,28). Finally, additional variables that might or have been shown to impact NAFLD, namely medications, diet, and comorbidities, will be closely monitored to examine the solitary effects of exercise on the gut and inflammation in the change of NAFLD within patients.

Therefore, the first study that will comprise the proposed research will provide novel data on the relationships between gut microbiomes, inflammatory levels, body composition, QoL, cardiovascular fitness, and muscular strength related to NAFLD severity. The second study will involve an exercise training intervention, examining the influence of exercise on those variables assessed in the first study – including potential changes on biomarkers relating to NAFLD severity. From these data, we aim to establish or add to the current research to advance our understanding of how exercise can be used to potentially treat and manage NAFLD.

5.2. Aims of Study

The proposed aims of this thesis are to examine:

i. Potential relationships between gut microbiome composition, inflammatory markers, and other clinical biochemical markers in a cross-sectional study of NAFLD patients;
ii. The influence of exercise training changes in gut microbiome and biochemical markers associated with inflammation in NAFLD patients.

5.3. Hypothesis

It is hypothesised that:

i. The gut microbiome composition is related to markers of chronic systemic inflammation and disease severity in NAFLD patients.
ii. Exercise training will elicit changes in gut microbiomes and systemic inflammation, which will be related to improvements in markers related to NAFLD.

5.4. Study Objectives/Expected outcomes

5.4.1. Primary outcomes

i. The findings will advance understanding of how exercise may improve outcomes for NAFLD patients through the mechanisms involved in gut microbiomes and inflammatory markers.

ii. Confirmation of the relationships will contribute to identifying the most effective type/s of exercise to improve health and outcomes for those with NAFLD – and potentially to prevent the progression of the disease for those at risk of its development.

iii. Monitor the change of gut microbiomes and inflammatory markers in relation to marks of liver disease severity/activity.

5.4.2. Secondary outcomes

i. Contribute to creating better diagnostic criteria regarding gut microbiomes when determining the severity of NAFLD.

ii. Assess potential changes in quality of life of NAFLD patients in response to exercise training.

6. Project Design

6.1. Study Overview

This research involves two studies. First, a cross-sectional study will investigate possible relationships between cardiovascular fitness, muscular strength, and body composition with various biochemical markers, gut microbiome composition and NAFLD severity. The second study will examine the influence of exercise training on potential changes to the gut microbiome and markers of systemic inflammation; these will be compared to changes in markers of liver health in patients with NAFLD. Participants involved in the first study will be invited to continue with Study 2.

Study 1: Cross-sectional study

The cross-sectional study will examine potential relationships between gut microbiome diversity, liver fibrosis and markers of systemic inflammation in NAFLD patients. In addition, cardiovascular fitness, muscular strength, and body composition measures will also be compared to the gut microbiome, systemic inflammation, and liver fibrosis. At least 46 participants will be recruited via hepatologists at SCUH, sample size calculations in section 6.2.3. Participants will undertake a comprehensive assessment including fitness, strength, body composition (DXA), gut microbiome composition (using faecal samples and next-gen sequencing) and inflammatory markers (via blood sampling and analysis at USC laboratory facilities at SCHI), and liver stiffness and steatosis with Controlled Attenuation Parameter (CAP) as shown in Figure 2.

Initial recruitment and delivery of the information packet to each participant will be conducted within the Liver Disease Clinic by Mr Christiaan Hattingh and Prof James O'Beirne. Informed consent to participate from patients will be confirmed with forms prior to attending Day 1 of initial assessments.

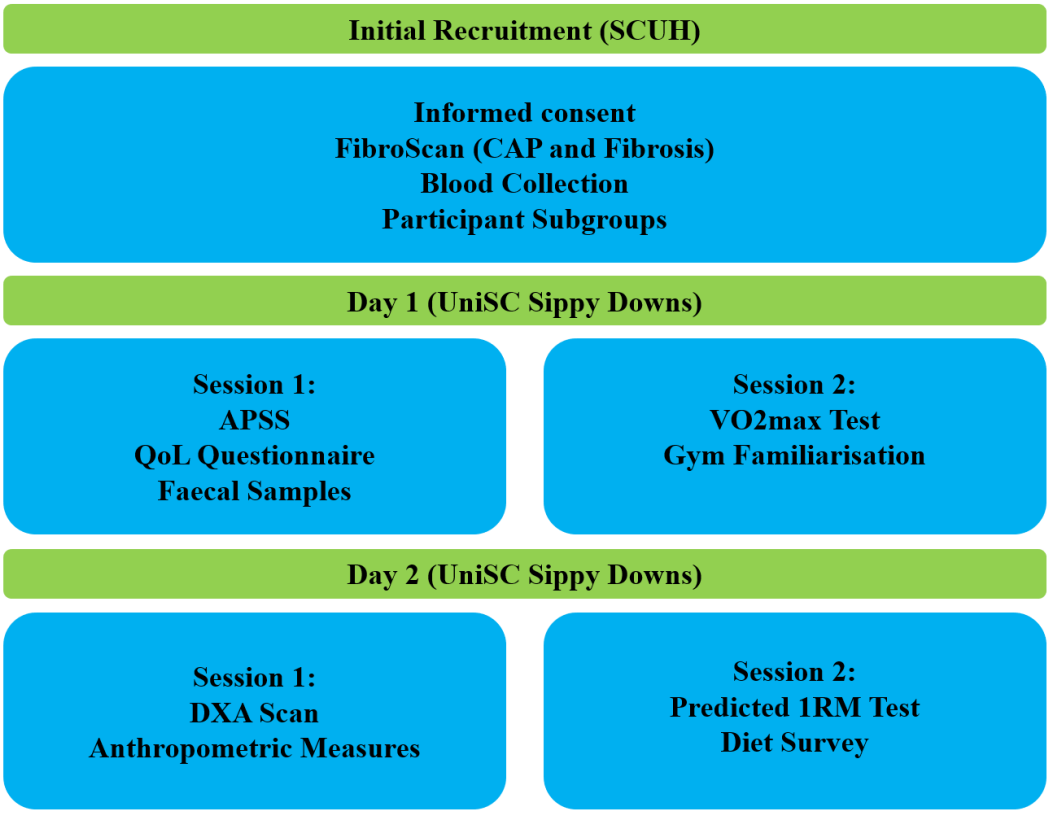

Figure 2: Outline of Study 1 initial assessment. (CAP: Controlled Attenuation Parameter, APSS: Adult pre-screening system, 1RM: one repetition maximum, DXA: Dual-energy x-ray absorptiometry, FM: Fat mass, FFM: Fat-free mass, Qol: Quality of life)

Patients will be asked to attend testing having abstained from alcohol and not smoked for 24 hours. They will also need to be 'rested', having avoided endurance exercise for 24 hours, and resistance exercise for 72 hours (outlined in 6.4.3.1). Though a light breakfast will be consumed be participants on the initial recruitment and Day 1 of testing, they will need to fast for 12 hours before the DXA scan on Day 2. Special consideration and exemptions will apply to participants who must manage their blood glucose levels on Day 2 for safety. Meals will be strongly advised before commencing Session 2 on Day 1 and 2.

Initial recruitment will involve identifying suitable participants attending the SCUH liver clinic by Prof James O'Beirne. These patients will be provided with an information pack and PICF (participant information and consent form) regarding this research project before attending their next appointment consisting of a FibroScan (liver stiffness and CAP) and blood collection. Collecting the outcome measures using the FibroScan is best when the participant is at rest as exercise increases hepatic blood flow which can artificially increase LSM.

Day 1, Session 1 will start with the pre-screen testing of each eligible participant using the APSS (Appendix A). Additionally, the participants will be asked to complete the Short form (SF)-36 QoL questionnaire, to enable assessment of quality of life (Appendix B). Participants will also be required to bring or provide a faecal sample during this session, using the outlined protocol in 6.4.3.2.3 (collection kit provided at initial recruitment appointment at SCUH). The break between the sessions allows the participants to have a light lunch before performing a VO_2max test, outlined in section 6.4.3.2.6, before attending a gym familiarisation session to help participants learn the correct and safe techniques of operating the relevant exercise machines before the 1RM test on Day 2.

Day 2, Session 1 will start with a DXA scan, with all participants required to be in a fasted state (i.e., no food consumed for 12 hours). Following the DXA scan, participants/patients will consume a light meal and rest for 30 minutes before commencing Session 2. Next, each participant will perform a predicted 1RMmax test to assess muscular strength, as outlined in section 6.4.3.2.7. The participant will also complete an online dietary intake survey. A qualified exercise professional will be present for all sessions in Study 1.

Study 2: Training (Intervention) Study

Following the first study, a 12-week exercise intervention will compare potential changes in the gut microbiome, body composition and biological markers of inflammation and liver stiffness following exercise in a cohort of NAFLD patients, in Study 2. Similar periods of training (≥ 12 weeks) have been shown to elicit significant changes in primary and secondary outcome measures (4,11). Participants will undergo a comprehensive assessment of the same outcome measures used in Study 1 at baseline (0 weeks), again at six weeks and on completion of the intervention of (post week 12), as shown in Figure 3. Diet will be assessed at the same three time points (0, 6 and 12 weeks); participants will be asked to maintain the same diet across the 12 weeks. The sample size will be informed by Study 1, but initial calculations show that 36 participants will be needed for Study 2.

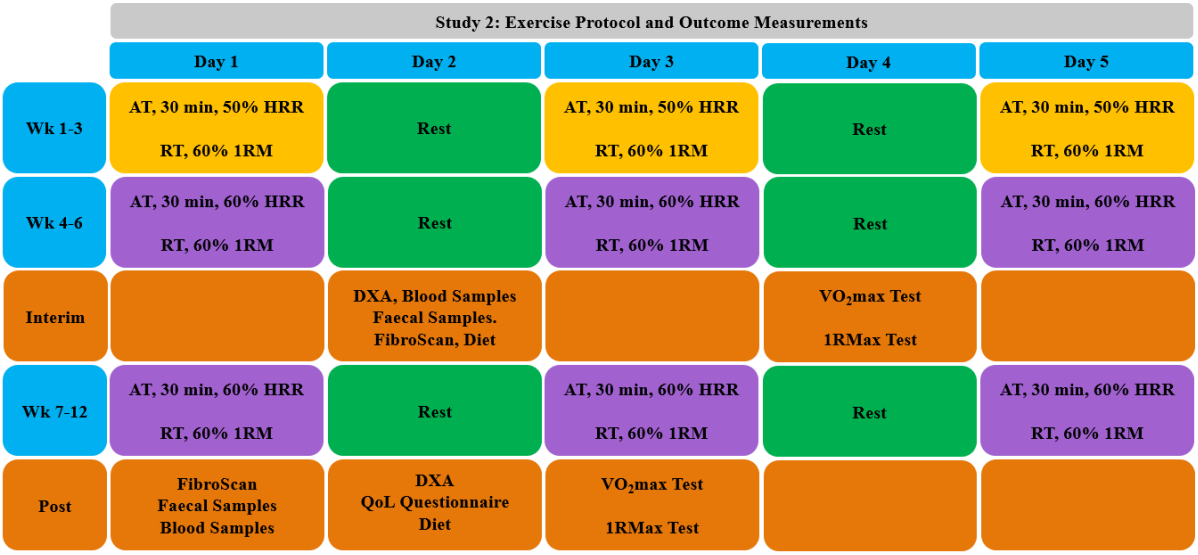

Figure 3: Outline of Study 2 intervention (FibroScan: Controlled Attenuation Parameter and Liver Fibrosis, 1RMax: one repetition maximum, DXA: Dual-energy x-ray absorptiometry, RT: Resistance training, AT: Aerobic training, HRR: Heart rate reserve).

6.2. Participants

6.2.1. Target Population

A hepatologist will categorise patients diagnosed with NAFLD into three subgroups, depending on severity and comorbidities (LSM <3, 8-12.5 and >12.5). The participants will be invited to participate in both cross-sectional and longitudinal studies. They will need to sign a participant informed consent form (Appendix C), a DXA consent (Appendix D), and an exercise pre-testing preparation checklist (Appendix E) before being accepted into the study. This study will also be open to Aboriginal and Torres Strait Islander (ATSI) people if they meet the inclusion and exclusion criteria, and if they want to participate. We will provide contact details for health liaison officers etc. to facilitate and address any ATSI needs to ensure an appropriate and respective experience for all participants.

6.2.2. Eligibility

Inclusion criteria:

The following criteria will need to be met before participants can be confirmed in the study.

1. A diagnosis of NAFLD
2. No other liver disease or cancer present
3. Other well-managed comorbidities, e.g. T2DM, and hypertension, are approved for the study
4. No recent or current exercise performed*
5. Commit to the cross-sectional study and assess for the longitudinal study
6. Complete consent form

Exclusion criteria:

The following criteria will disallow participants from taking part in the study.

1. Younger than 18 years of age
2. Serious cardiovascular, respiratory, or neurological diseases and cancers
3. Poorly controlled hypertension, glucose regulation or other serious chronic diseases
4. A disability that could prevent the completion of exercises
5. High-risk participants with contraindications to exercise
6. Currently exercising more than 60min*
7. Participation in conflicting studies
8. Participants with internal artefacts that may obscure DXA readings
9. Participants who are pregnant, attempting to conceive or lactating.
10. Participants exposed to radiation levels deemed unsafe in the DXA pre-testing preparation checklist.
11. Participants who cannot complete the 1RM and VO2max protocols.
12. Medications that could alter outcome measures**.

*Current sedentary lifestyle with >60 min of exercise performed weekly.

**Will be discussed with Prof James O'Beirne to confirm current medications prescribed to patients who have met the inclusion criteria of this research study.

6.2.3.Sample Size

Study 1:

Sample calculation is determined through matched grouped t-test (G*Power) of relevant literature with regards to outcome measures with the highest predicted total sample used (29). Mean (SD) of the control and experimental group were used to calculate effect size. A one-tailed T-test is then performed with Alpha: 0.05 and Power 0.8 to calculate sample size required. Additionally, several other journal articles have reported significant change in key outcome measures with regards to their sample size (Table 1).

Dropout rates for single test cross-sectional study is predicted to be low. Although, an additional 15% will be accounted for and recruitment numbers will be adjusted to meet the statistical significance during data collection. Therefore, a total of 46 participants (15% dropout included) will be recruited based on calculations in Table 1.

Statistical analysis of gathered data will include a multivariate linear regression. T-test with matched groups will be used to determine any potential correlation present between the key outcome measures Section 6.4.3.2 in the master ethics document.

Table 1: Sample size sources for the cross-sectional study

Reference	Outcome measure	Control (Standard deviation)	Diseased population (Standard deviation)	Effect size	Total sample size
(29)	ALT	51.1 (5.3)	36.8 (5.2)	2.723686	36
	Firmicutes	0.25 (0.075)	0.18 (0.06	1.398799	26 (estimated with p<0.001)
	BMI	24.8 (5.2)	31.3 (8.9)	0.8917926	34
	Triglycerides	37.6 (55)	148.2 (61.5)	1.895764	10
(30)	Proteobacteria	1.06 (0.94)	0.65 (0.63)	0.4941912	27

In addition, data reflected a p <0.001 for steatosis (%) and fibrosis (F0-4) between controls and diseased population in a cross sectional study with 75 participants (29).

Study 2:

Sample size will be determined through matched pairs t-test in G*Power from literature with the relevant outcome measures over two time points (pre and post) in Table 2. However, for three timepoints (pre, midway, post) 'Power analysis with simr' will be used for sample size calculation. Mean (SD) of the baseline and post experimental group were used to calculate effect size. A one-tailed T-test is then performed with Alpha: 0.05 and Power 0.8 to calculate sample size required.

A dropout rate of 30% will be accounted for and recruitment numbers will be adjusted to meet the statistical significance during data collection. Therefore, to consider all primary outcome variables, a sample size of 36 total would be sufficient to account for a 30% dropout.

Study 2 will have a linear mixed effects model performed for the final collected data. The following equation will be used: y~ + time + (1/id) + error

Table 2: Sample size sources for longitudinal intervention

Reference	Outcome measure	Baseline measure (Standard deviation)	Post measure (Standard deviation)	Effect size	Total sample size
(26)	CAP	359.75 (38.63)	275.00 (71.38)	1.412408	5
(31)	ALT	38.9 (4.6)	33.9 (3.8)	1.174602	7
	AST	27.5 (1.5)	25.3 (1.5)	1.174273	7
(10)	VO2peak,mL/kg/min	23.6 (0.9)	25.1 (1.1)	1.477994	5
(32)	Ferritin	158.7 (30.7)	138.4 (26.8)	0.7012644	15
(33)	Fibrosis (kPa)	7.4 (4.2)	5.5 (2.4)	0.5205968	25
(30)	Proteobacteria	1.06 (0.94)	0.65 (0.63)	0.4941912	27

Gut microbiome data is often not normally distributed and data either will be normalised using log-transformation or studied using non-parametric statistics. Well-established analysis pipelines will be used in the analysis of the gut microbiome including HuMANn3 and MetaPHLaN which include correction for multiple testing. The gut microbiome data can be entered into multivariate statistics. Gut microbiome statistical analysis to be used to determine any correlation to disease severity in Study 1. Whereas changes in microbiome diversity, composition and functional capacity will be monitored during Study 2 – also to determine whether a correlation exists with the improvement of liver disease makers.

The final analysis for both studies will be either a t-test for normal distribution or Wilcox for non-normal distribution. Both studies will use either a t-test for parametric data or Wilcoxon rank singed test for non-parametric data.

This study will consist of a within-control group and therefore do not require an additional control group. Additionally, this research investigation is designed to be a pilot study.

Therefore, since this is novel data, our focus will be on the base line characteristics of our variables (Study 1), to further our understanding of the relationships. With this knowledge, potential outcomes are improved diagnosis for NAFLD patients and better determination of severity of the disease. Since we are approaching this research as a pilot study, we used power calculations to estimate the number of participants needed to gather statistically significant data.

The longitudinal exercise intervention (Study 2) will then monitor the effects of exercise on gut microbiome alpha diversity and key liver disease markers (steatosis, liver stiffness, inflammation, liver serum markers) on participants of the first study. Any significant changes to either or both gut microbiome and liver disease state will be attributed to the exercise intervention. In this research project, data gathered from Study 1 will serve as a type of "control" for Study 2 effects. Extensive literature reviews will be undertaken to gather information on baseline characteristics of NAFLD patients with regards to baseline gut microbiome alpha diversity, presence of inflammatory markers, body composition, liver disease state (fibrosis, steatosis, liver enzymes etc) as well as the impact of exercise on this population. With the information gathered from past credible research and Study 1, we can confidently proceed with Study 2 without a control group, since we know the effects of exercise (extensively researched) on NAFLD, with the novel variable being gut microbiome diversity.

6.2.4. Participant Recruitment

Participants will be recruited by Prof James O'Beirne via the liver disease clinic SCUH from January 2023.

6.2.5. Confidentiality

Unique codes that de-identify participant data will be used and all data will be securely stored in password-protected computers and locked filing cabinets within the Chief investigator's office. All material will be archived for a minimum of seven years following completion of the trial. No individual will be identifiable in any reports and publications.

6.3. Ethical Considerations Concerning Study Inclusion

This study will conform to the principles of the Declaration of Helsinki according to international standards of Good Clinical Practice (GCP) guidelines, applicable to Australian Government regulations and Institutional research policies and procedures. The UniSC Human Research Ethics Committee (HREC) will approve this protocol and any amendments before study commencement.

6.3.1. Informed Consent

Participants who are eligible and express an interest in the study will receive and be asked to read a detailed *Participant Informed Consent Form* (PICF; Appendix C). This document provides information regarding the requirements of study involvement (e.g., time commitment, details of exercise testing sessions, risks etc.).

In the consent form, it will be explained that:

a) Participation in the project is entirely voluntary.
b) Participants will be free to withdraw from the study without penalty – even after providing written informed consent.
c) Participants will not be identifiable from published data and findings.

Participants will only be asked to sign the consent form once they understand what the study involves, have all their questions answered to their satisfaction and are willing to participate in the research.

The *PICF* will be submitted with the protocol for review and approval by the HREC, with all documents printed on official UniSC letterheads. The formal consent of a participant, using the HREC-approved consent form, will be obtained before the participant is submitted to any study procedure. If any modifications to the study design (e.g. further follow-up assessments) or the intended use of data are proposed and ethically approved by the HREC, a revised *PICF* outlining these changes will be provided to the participant, and written consent will be required again.

6.3.2. Participant payment and reimbursement

Participants will not receive any reimbursement or remuneration for their participation. However, there will be numerous health benefits associated with this study and no out of pocket expenses will incur on the participants. See section 6.4.6 in this document.

6.3.3. Voluntary participation and withdrawal

All invited participants will have it explained that their involvement in this research project is voluntary, both verbally and in the recruitment process and in writing. Participants will be informed they can withdraw from the study at any time, without penalty. Participants will also be able to contact project staff for further information before choosing whether to participate. Upon withdrawal, participants in the study will complete a *Withdrawal of Consent* form (part of the PICF, Appendix C). They can request that all data relating to them be removed from the study and not included in data analysis. However, if appropriate, before complete withdrawal, participants will be asked to have the outcome measure of the study remeasured for a final data point.

6.3.4. COVID-19-related considerations

All participant contact, assessment measures and procedures will involve social distancing and heightened sanitisation by the UniSC COVID-safe plan and Queensland Health COVID-safe requirements when on a Queensland Health Site. Thorough cleaning and sterilisation of all study testing locations and equipment will be carried out between participant assessments. Research team members will wear gloves, and social distancing rules will be followed where not otherwise required for data collection. Before attending their assessments, all participants will be contacted to confirm they are not experiencing flu-like symptoms, are not awaiting COVID-19 test results, and have not recently travelled from a designated hot spot. Upon arrival for their assessment, participants will again be asked standard COVID-19 screening questions and be asked to sanitise their hands using an alcohol rub. These measures will be revised to comply with the current UniSC COVID-safe policies and guidelines provided by the Queensland Health authorities.

6.4. Research Activities

6.4.1. Project Timeframe and Participant Commitment

Study 1 will run for one week per participant.

Study 2 will be a longitudinal study with participants recruited from study 1, consisting of 12 weeks of supervised exercise. Additional time will be needed before and after the intervention for sample collection, totalling an 14 to 16-week commitment

Both studies are expected to commence in early 2023.

6.4.2. Participant Screening

After eligible patients are recruited from the NAFLD clinic by Prof James O'Beirne, additional eligibility requirements will be assessed through inclusion and exclusion criteria. This includes a medical history to determine if they can safely undertake the assessment measures involved in the study. Suitable participants will be screened using the adult pre-exercise screening system (APSS) to identify any risks or limitations to performing exercise. If a participant indicates 'yes' to Part A of the

questionnaire, they will be asked to seek clearance to participate from a doctor. Part B of the questionnaire seeks additional information about potential cardiovascular risk factors. This will enable the researchers to determine the relative level of risk of a cardiovascular event during exercise and exclude participants with significant risk factors based on the American College of Sports Medicine Guidelines (34).

6.4.3. Testing Procedures

6.4.3.1. Pre-testing preparation

Participants will be provided with standard pre-testing preparation information. They will be asked to confirm their adherence by completing and signing a pre-testing preparation checklist before each testing session (Appendix E). Participants will be required to be well-hydrated upon arrival (have 1-2 cups of water one-hour preceding testing and exercise), avoid planned endurance exercise for the 24 hours preceding assessments, avoid resistance exercise three days preceding each assessment, and avoid caffeine, alcohol, and tobacco for 24-hours before the assessment. Additionally, participants will be asked to wear non-restrictive clothing and enclosed footwear and to consume a light breakfast an hour before the exercise sessions.

6.4.3.2. Primary and secondary outcome measures

6.4.3.2.1. Liver fibrosis

Liver fibrosis will be measured through vibration controlled transient elastography (VCTE) using a FibroScan machine. This non-invasive procedure has been validated to give accurate liver stiffness readings which correlate with the degree of fibrosis (26). The participants will be asked to lie down on a medical assessment bed. The Fibroscan probe will be lathered with gel to reduce impedance and reflection between the device and the patient's skin. Timepoints of scans will include one in Study 1 and two in Study 2, conducted in weeks 6 and post week 12.

6.4.3.2.2. Controlled attenuation parameter

Controlled attenuation parameter (CAP) measures liver fat content to determine the grade of steatosis in dB/m. This outcome measure will also be collected using the same FibroScan device in subheading 5.4.3.2.1 for Liver fibrosis. This data collection method is also non-invasive and has been shown to detect all grades of hepatic steatosis. This is a convenient tool for NAFLD interventions, with real-time monitoring of liver fat content change throughout the study (1). The participants will be asked to lie down on a medical assessment bed. The Fibroscan probe will be lathered with gel to reduce impedance and reflection between the device and the patient's skin. Timepoints of scans will include one in Study 1 and two in Study 2, conducted in weeks 6 and post week 12.

6.4.3.2.3. Gut Microbiomes

To assess gut microbiomes, 200mg of faecal samples will be collected at the participants' home with the provided collection materials. Samples will be processed at UQ to determine bacterial type,

quantity and diversity. Final products will be stored by research team members in a -80°C freezer at SCHI. Only one faecal sample will be collected in Study 1. In Study 2, samples will be collected on three occasions: at baseline, at week 6 (midway) and after the completion of week 12. Restricted access to the sample material and de-identified data will be put in place to maintain participant confidentiality.

6.4.3.2.4. Blood biochemistry

In both Study 1 and at two timepoints in Study 2 (baseline, midway and after week 12), 2 serum separator and 1 EDTA tubes (20-30mL) of blood will be sampled from an antecubital vein using a 21-guage cannula, by a qualified phlebotomist, at UniSC. The samples will be used for analysis of GGT, HbA1c, total cholesterol, HDL, LDL, triglycerides, AST, ALT, ferritin, adiponectin, interleukin-1β, high-sensitive C-reactive protein (hs-CRP), TNF-α, IL-1, IL-6, IL-10.

Samples will be stored on ice until preparation, after which plasma and serum samples will be centrifuged for 10-minutes at 3000 rpm following a 20-minute coagulation period. Serum and plasma (EDTA) samples will be pipetted into individual aliquots and then stored at –80 ºC before analysis. The QuantiFERON-TB Gold Plus blood collection tubes (or similar products) will be used for blood sample collection and storage. The blood samples will be used to look at inflammatory markers and cytokine changes relating to exercise using the QIAGEN Multi-Analyte ELISArray Kits (or similar). The processing of samples will be completed at the SCHI lab.

6.4.3.2.5. Body composition

Anthropometric measures including height, weight, calculated BMI, waist circumference and waist-to-hip ratio will be recorded using the ISAK protocols (35). Additionally, a qualified operator will use the Dual x-ray absorptiometry device to collect data using the 3-compartment model, consisting of fat mass, fat-free mass, and water. The DXA (GE Lunar iDXA (GE Healthcare, Madison, WI), located on level one of the Sports Tower (T1.05), UniSC, will be used. Scans will be analysed by a trained operator using manufacturer-supplied software (GE enCORE v.13.60 software GE Healthcare and the Geelong reference database). Calibration will also be completed per the manufacturer's recommendations. Additionally, there is a minimal risk of radiation exposure to participants during this scan which is covered in section 6.5.3.

6.4.3.2.6. Peak aerobic capacity: VO_2peak

To assess cardiovascular fitness, participants will complete a peak aerobic capacity or VO_2peak test using a maximal graded exercise test to volitional fatigue using the Bruce protocol on a treadmill (36). The Bruce protocol is comprised of multiple exercise stages of three minutes each. At each stage, the gradient and speed of the treadmill are elevated to increase work output, called METS. Stage 1 of the Bruce protocol is performed at 2.7 kilometres per hour (kph) and 10% gradient. Stage 2 is 4 kph and 12% incline, while Stage 3 goes to 5.5 kph and 14% incline, and if required, Stage 4 at 6 kph and 16%

grade incline. Expired oxygen and carbon dioxide will be continuously analysed during exercise via indirect calorimetry (TrueOne 2400, Parvomedics Inc, Utah, USA), and V̇O2peak, will be determined as the highest average recorded volume of fractional expired oxygen and carbon dioxide during the final stage of the test. This information will inform the longitudinal intervention's benefits of aerobic exercise during Study 2. This test will be remeasured during Study 2 (weeks 6 and 12) to determine cardiovascular fitness improvements. Further information regarding the aerobic exercise protocol is provided in section 6.4.5.1.

6.4.3.2.7. Strength: Predicted 1RM

The participants' strength will be predicted using a 3-5 repetition max (RM) test to estimate the 1RM. This test will be performed in the UniSC gym facilities. Participants will be made familiar of all exercises performed on Day 2 of Study 1. The exercises are described in section 6.4.5.2, which will primarily be used for Study 2. A warm-up set will start the protocol before additional resistance is added until only a maximum of five repetitions can be performed for every exercise. This test will determine the intensity of resistance training by using the percentage of 1RM for each participant in Study 2.

6.4.3.2.8. Quality of life questionnaire

QoL is shown to be linked to the histological severity of NAFLD using the SF-36 (37). The primary outcomes are the correlation between fibrosis stage with relation to the eight sections (physical function, physical limitations, pain, general health, energy, social function, emotional limitations, and emotional well-being) of SF-36 score. This questionnaire will be completed by participants during Study 1 and at each timepoint in Study 2.

6.4.4. Control parameters.

6.4.4.1. Physical activity

Participants will be asked to avoid resistance exercise for three days and endurance exercise 24 hours before each testing session. Each must confirm their adherence by completing and signing a pre-testing preparation checklist before each testing session (Appendix E).

6.4.4.2. Diet

Participants will be asked to consume the same (or very similar) light meal at least one hour before each exercise session in Study 1 and 2. These meals will be recorded on the pre-testing preparation checklist before each testing session. Therefore, participants must confirm their adherence by completing and signing a pre-testing preparation checklist before each testing session.

An online dietary intake survey (Australian Eating Survey) will be used to determine the typical food intake of each participant for Study 1. Throughout Study 2, diet will be monitored with a 3-day dietary intake record (at 0, 6 and 12 weeks) to ensure no signification deviation from current eating

habits and macronutrient intake (Appendix F). A significant change in diet, especially carbohydrates, fat and fibre, has the most significant impact on physiological mechanisms affecting gut microbiome composition (15,16). The data gathered from each participant will be transferred and monitored with the FoodWorks software application and stored securely on UniSC drives (refer to 6.6 Data Management).

Although diet alone has shown to improve NAFLD outcomes, this research project will not involve dietary manipulation. Instead, we will be looking to ensure that each participant's diet across the training period does not significantly change.

6.4.4.3. Alcohol consumption

Abstinence or consumption below the NAFLD classification threshold of alcohol must be adhered to for the duration of the study.

6.4.4.4. Drugs

Drugs such as GLP-1 analogs and the SGLT2 inhibitors could potentially affect AST/ALT liver markers. New diabetic medications can affect the liver positively and will be documented. Prof James O'Beirne will determine the potential impact the participant's current pharmacological treatment on study outcome measures during the initial recruitment stage and throughout Study 1 and 2.

6.4.5.Exercise Intervention

A combination of aerobic and resistance training has been proven to yield the most significant physiological changes in outcome measures of NAFLD patients compared to the implementation of these exercise modes individually (4,9,11).

Study 1 will be a precursor to Study 2 with exercise familiarisation and starts at lower moderate intensity and will progress to higher-moderate intensity for the 12 weeks of intervention for both aerobic and resistance exercise. The median duration of exercise protocols in studies was 12 weeks. However, evidence suggests that a longer training period will likely lead to greater change (1,4,11,22), with shorter eight weeks of RT not yielding significant remedial effects on NAFLD (10). Combined exercise is also clinically relevant to improving overall health from a clinical treatment perspective.

6.4.5.1. Aerobic Exercise

Aerobic exercise has been proven to reduce the risk and improve liver health in NAFLD patients (11). In addition, biochemical markers related to NAFLD, including triglycerides, AST, ALT, total cholesterol, LDL, and HDL, have been reported to significantly improve using aerobic exercise interventions (11,22). Other health benefits are a reduction in BMI, waist circumference, intrahepatic lipids, and significant improvements in liver steatosis (1,11,22).

The 12-week longitudinal exercise intervention will include a combined aerobic and resistance training protocol. The outline of the aerobic exercise protocol is shown in Figure 4.

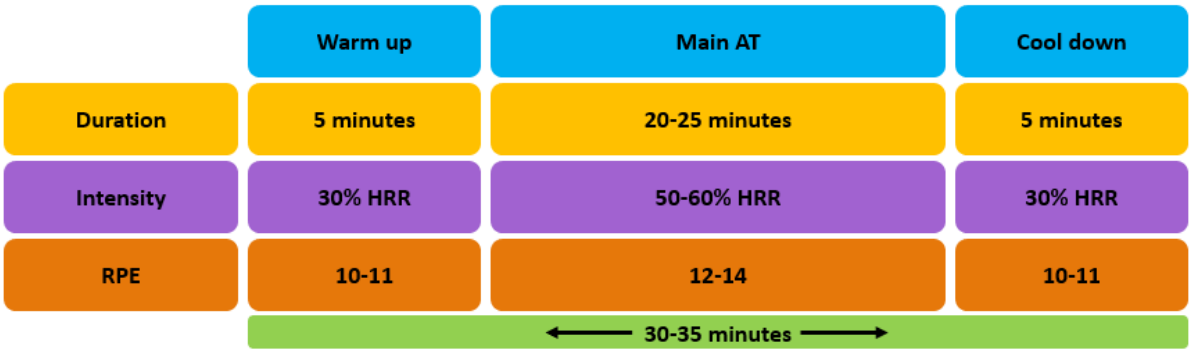

Figure 4: Aerobic exercise protocol (HRR: Heart Rate Reserve, AT: Aerobic Training, RT: Resistance Training, RPE: Rate of Perceived Exertion).

The duration and intensity of the core aerobic exercise protocol are based on the America College of Sports Medicine (ACSM) guidelines (34). Heart rate reserve (HRR) will be used to calculate exercise intensity in this study. HRR is an accepted and appropriate method for each participant to attain relative exercise intensity. 50-60% HRR is categorised as moderate intensity, with similar hepatic benefits, less risk of injury and increased compliance demonstrated in studies compared to high-intensity aerobic exercise (1). Multiple studies have demonstrated that increased frequency of aerobic exercise, to at least three days a week, significantly increases the benefits associated – representing a dose-response relationship with exercise and liver health (1,22). The exercise frequency for this protocol will be three days a week with a minimum of one rest day between exercise days. Exercise duration responds similarly to frequency, with longer durations yielding more benefits. The aerobic component of the protocol will last a total of 30-35 minutes. The treadmill/bike will be the chosen mode of exercise (participant preference).

6.4.5.2. Resistance Exercise

Resistance training is the other exercise type used in this combined intervention with evidence promoting its effectiveness and benefits for the treatment of NAFLD (1,11,12,22,26). Studies have shown significant changes in triglycerides, total cholesterol, AST, ALT, and serum ferritin in response to resistance training. Additionally, improvements in steatosis, body weight and waist circumference have been shown (1,11,12,22). Furthermore, although more expensive to implement, resistance training appears to improve liver health with less energy consumption than aerobic exercise (12,26). The protocol for the resistance training intervention of the longitudinal intervention is shown in Figure 5.

Stage	Warm up	Main RT
Duration	1 Set	16 repetitions @ 40-50% 1RM (Major muscle groups)
Intensity	3 Sets	10-12 repetitions @ 60-70% 1RM (Can progress to 80% 1RM)

Figure 5: Resistance exercise protocol (RT: Resistance Training, RM: Repetition Maximum).

The exercises chosen for the intervention will all be machine-based for safety. Exercises will include: Leg press, Lat pulldown, Chest press, Leg curl, Seated row, Shoulder press/lateral raises. The exercise intensity/weight and sets are based on the ACSM guideless. The frequency of resistance training will match the aerobic exercise protocol, with studies demonstrating significant improvement in results for NAFLD patients, and will be performed on the same day (1,22).

6.4.6.Study Benefits

Benefits to participants taking part in this research include:

1. Gut microbiome and inflammatory marker assessment

Participants will receive a gut microbiome assessment through faecal samples provided in Study 1. This assessment is not a common test performed in a general health check-up and may provide insight into their current lifestyle's impact on gut health. In addition, inflammation is also a key element of the disease. Additionally, this assessment may clarify the extent of the impact inflammatory markers have on the participant's liver disease.

2. A body composition assessment.

Participants in the study will receive individual feedback from the body composition assessment, including DXA scans. Access to this assessment in clinical and public health settings is often costly and associated with long waiting periods. Many participants may experience some benefit from accessing these services within this research-based context.

3. Introduction to different types of exercise.

Participants will receive expert instruction in exercises that they may be interested in but have not had the opportunity to experience. In addition, familiarisation in a gymnasium setting will allow the participant to feel confident with basic exercises and are more likely to start/adhere to a program.

4. Potential improvement of NAFLD

Research has shown that exercise improves liver steatosis, reduces inflammation related to NAFLD, and improves gut dysbiosis. These combined effects are likely to manifest during the 12-week training intervention.

6.5. Study Risks and Risk Management

The pre-screening and information pack regarding the study will explain that there are minimal risks associated with participation in the research proposed. The procedures used are non-invasive and are standard in exercise physiology research. This study specifically targets the population group and the practicality of real-world applications. Nonetheless, the risks of participating in the present study include:

6.5.1. Risk of Cardiovascular Event

The VO_2max and predicted 1RM tests used in this protocol are deliberately challenging. The tests may be confronting and intensive to some participants in this population, as they are designed to push participants to their best level of performance. As a result, some participants may experience general performance anxiety or distress associated with testing. With qualified clinical sports scientist supervision, participants will be encouraged to perform their best throughout the maximal test to minimise the potential for distress and optimise results. The protocol will be explained before and regularly guided throughout each test with increasing difficulty until their best performance is achieved. With years of exercise experience, the researchers will be aware of anxiety, distress or frustration symptoms and provide appropriate assistance to the participant if needed.

All exercise has some degree of risk, notably fatal and non-fatal cardiovascular events. However, the general medical consensus on the risks versus benefits of exercise in adults is that the benefits of exercise far outweigh the risk of an adverse cardiovascular event during and immediately after exercise.

To minimise risk, this study will:

- Have participants complete a APSS risk questionnaire before acceptance into the study to confirm the level of risk that may restrict their capacity to complete the assessment measures.
- Involve the continuous monitoring of heart rate and perceived exertion throughout exercise and recovery. In the rare event that complications may arise during the protocol, safety procedures outlined at UniSC gym will be followed.
- Ensure that all exercise performed in this study is supervised by qualified exercise professionals.
- Exercise supervisors will have CPR accreditation.
- Participants will be monitored for 15 minutes after testing to ensure they have sufficiently recovered before leaving the testing and training facilities.

6.5.2. Risk of Injury, Muscle Fatigue and Soreness

There is a risk of participants sustaining injuries such as acute muscle strains. However, exercise professionals will significantly reduce these risks through careful supervision and monitoring of participants throughout each protocol.

Delayed onset muscle soreness (DOMS) is often experienced after exercise testing/training, especially in relatively untrained or sedentary individuals. Participants will be informed of this possibility and educated about the symptoms and effective methods for preventing and relieving DOMS. Warm-up and cool-down procedures are essential strategies for preventing DOMS and will be incorporated into each testing and training session. This will reduce muscle soreness and assist participant recovery towards a resting state before leaving the session.

6.5.3. DXA: ionising radiation risks

This research study includes DXA whole body scans (for assessing muscle, fat and bone mass). DXA is a routine measurement for bone density and body composition. The dose associated with DXA (GE Lunar iDXA) will be 3 µSv for the whole body. In comparison, an individual receives between approximately 4-5.5 µSv for daily natural background exposure, 80 µSv for a return trans-Pacific flight, 100 µSv for a chest x-ray, and 2000 µSv for a lumbar spine x-ray. Therefore, although ionising radiation is used in the scan, the amount of radiation is below the dose incurred on a trans-pacific flight, and the corresponding risk from participating in this study is low.

The School of Health and Behavioural Sciences at UniSC operates standard risk management procedures for staff conducting multiple scans, which have been approved by the institutional Radiation Safety Officer, Dr Ava Farley. The research team is familiar with the ARPANSA Code of Practice "Exposure of Humans to Ionising Radiation for Research Purposes" (Appendix G). Appendix H contains the Research Study Radiation Dose and Risk Assessment outlining dosages relayed from the School of Health and Sport Sciences DXA (GE Lunar iDXA (GE Healthcare, Madison, WI)), located on level one of the Sports Tower (T1.05), and Appendix I outlines our Certificate of Compliance. In addition, the investigators have obtained an assessment and approval for the use of DXA Ionising radiation in this project from the School of Health and Sport Sciences local radiation safety officer, deeming that the radiation is appropriate for use at UniSC and complies with ARPANSA Codes of Practice (Appendix G). If the committee has any concerns, Dr Ava Farley may be contacted by phone on 07 5459 4605.

The student investigator has completed all required DXA training specific to GE Lunar iDXA machines and is accredited by ANZBMS to operate DXA scanners for the ANZBMS Certificate of Completion in clinical bone densitometry for the Student investigator (Appendix J). A license to

operate the USC DXA machine will be granted after ethics approval is secured and training is completed. Dr Mia Schaumberg, the Co-supervisor, is licensed to operate DXA machines and can assist the student investigator where necessary. Appendix K for the current DXA operation license of the Co-supervisor, Dr Mia Schaumberg.

Before scanning, information on previous exposure to radiation will be collected from participants (X-Ray imaging scans in the previous 12 months or working with a source of radiation as part of their occupation) to ascertain suitability for this study (Appendix D). Additionally, as DXA scans can be dangerous to unborn babies, females who are; pregnant, conceiving, or lactating will be excluded. If participants are unsure if they are pregnant, they will be asked to have a pregnancy test first to clarify.

6.5.4. Blood sampling risks

6.5.4.1. Risk of bruising and infection associated with venous blood sampling

A qualified phlebotomist will sample all blood to minimise any risk of bruising, and all occupational health and safety procedures will be followed to minimise the risk of infection associated with venous blood sampling. Venous blood sampling is a minimal-risk, routine procedure for numerous exercise physiology studies conducted at the UniSC. All blood sampling procedures will be carried out in dedicated laboratory spaces, and risk assessments have been completed for these procedures.

6.5.4.2. Risks associated with providing biological samples

Though highly unlikely, a breach of confidentiality could occur during data collection or the biological sample collection procedures. It could also occur when the data and samples are stored at USC. The research team ensures strict security measures are used to protect information derived from biological samples carefully. Data and biological samples will be stored following the Australian Privacy Guidelines (April 2014) and the NHMRC National Statement on Ethical Conduct in Human Research (2007). It is highly unlikely that a third party will gain access to biological samples or data.

6.5.5. Disclosure of personal information

Participants will be asked to provide information on their medical history, which may make some participants uncomfortable disclosing personally sensitive information. Participants will be assured that their data will be stored in accordance with the Australian Privacy Guidelines (April 2014) and the NHMRC National Statement on Ethical Conduct in Human Research (2007). It is highly unlikely that a third party will gain access to this data.

6.5.6. Adverse Events

An adverse event is any untoward medical occurrence in a participant undergoing an intervention. The reporting period will be defined as the period from initiation of the study treatment to the end of the final intervention. A detailed review of each participant's medical history will be conducted at the point of initial recruitment and subsequent review. Information sought will aim to identify previous

procedures or hospitalisations and planned procedures/hospitalisations. Participant responses will be documented on their file, and the event monitored, with the appropriate action or medical referral taken should the matter be considered an ongoing or adverse event.

The Chief investigator will immediately inform the ethics committee if an adverse event occurs during the study, whether during an assessment with research staff or during an exercise session. All adverse events will be reported as per Standard UniSC Procedures. In a medical emergency, research team personnel will follow the standard UniSC procedures outlined. Adverse event reporting, including classification, grading and causality assessments, will follow standard NHMRC guidelines.

6.5.7. Quality control

The investigators will perform this research study according to this protocol, guidelines for GCP and the applicable regulatory requirements. The Chief Investigator will allocate adequate time to perform quality control activities, verify study data and ensure data are complete and accurate. This will be completed promptly. In addition, study-related procedures and processes, including adherence to standard operating procedure (SOP), will be monitored to ensure alignment with the approved protocol, GCP and UniSC guidelines.

6.6. Data Management

6.6.1. Data collection procedures

All data collected from participants will be de-identified, associated only with each participant's unique code. Data collection will be conducted according to the SOP manual. Inter- and intra-tester reliability will be assessed for all research team members involved in the data collection procedures (Appendix L).

6.6.2. Source documents

Reported study data will be verifiable from the source documents. Source data includes all information, original records, observations, or other activities necessary for the reconstruction and evaluation of the study. Source data are contained in the source documents. These original documents and data records include laboratory notes, participant diaries, case report forms, completed questionnaires and recorded data from automated instruments.

6.6.3. Record storage and retention

Any data collected as a part of this research project will be stored securely as per UniSC's Research Conduct Governing Policy and Research Data Management Procedures. The management and retention of physical and electronic data will follow a UniSC Research Data Management Plan (Appendix L) and be kept under safe storage for the duration required by University regulations. Such personal information will only be disclosed with your written permission or as may be required by law.

Confidentiality will be maintained at all times. Data will be stored securely in password-protected files in a de-identified format, with participant details and coding kept separately from responses and data. Physical trial files and documents will be kept in a secured filing cabinet within the Chief Investigators' office. The storage area is locked and accessible only to staff at UniSC who have security access.

6.6.4. Auditing and inspecting

The investigators will permit study-related monitoring, audits, and inspections by the governing HREC of all study-related documents (e.g. source documents, regulatory documents, data collection instruments, study data etc.) and study facilities (e.g. laboratories).

6.6.5. Privacy and treatment of personal information

The University, Investigators and project staff are bound to undertake this research under the Australian Privacy Principles 2014, the Australian Code of the Responsible Conduct of Research and NHMRC National Guidelines for the Ethical Conduct of Human Research (2007). They will take all reasonable measures to protect the confidentiality of participant records, and the identity of participants will not be revealed in any publication that may result from this study.

7. Statistical Analysis

7.1. Data Analysis

Data will be analysed using Microsoft Excel 2007, SPSS (version 22.0, SPSS, Inc., Chicago IL USA) and Prism® (Version 7.0, GraphPad, Inc., San Diego CA USA) statistical software packages. Specific data analysis procedures will be appropriately selected to answer each research question.

7.2. T-test analysis

Study 1: Statistical analysis of gathered data will include a multivariate linear regression. T-test with matched groups will be used to determine any potential correlation present between the key outcome measures (Section 6.4.3.2).

Study 2: Study 2 will have a linear mixed effects model performed for the final collected data. The following equation will be used: y~ + time + (1/id) + error.

Gut microbiome data is often not normally distributed and data either will be normalised using log-transformation or studied using non-parametric statistics. Well-established analysis pipelines will be used in the analysis of the gut microbiome including HuMANn3 and MetaPHLaN which include correction for multiple testing. The gut microbiome data can be entered into multivariate statistics. Gut microbiome statistical analysis to be used to determine any correlation to disease severity in Study 1. Whereas changes in microbiome diversity, composition and functional capacity will be

monitored during Study 2 – also to determine whether a correlation exists with the improvement of liver disease makers.

The final analysis for both studies will be either a t-test for normal distribution or Wilcox for non-normal distribution. Both studies will use either a t-test for parametric data or Wilcoxon rank singed test for non-parametric data.

7.3. Correlation analysis

Pearson's correlation coefficients or the non-parametric equivalent will be used to determine associations between appropriate variables of interest.

8. Results, Outcomes and Future Plans

8.1. Dissemination and Publication of Results

Data will be published in peer-reviewed journals and presented at scientific meetings and conferences. All publications and presentations will only report aggregate de-identified data, with no identifiable information published.

8.2. Feedback of Results to Participants

Individual research outcomes, including performance measures and fitness data, will be available to participants upon request, along with a general explanation of the study findings and results. In addition, the Chief Investigator may meet with the participant to discuss results and provide a summary of findings for the participant to discuss with their personal General Practitioner if assessment results are abnormal and may indicate an underlying health condition. Study outcomes, whilst informative, are primarily research outcomes only and cannot 'diagnose' any conditions. To minimise the influence of confounding variables and bias on study outcomes, participants will not be provided detailed feedback regarding their performance during assessments.

8.3. Project Closure Processes

At the time of submission, the project timeline estimates that participant data collection will be completed by the end of 2023. At the closure of data collection, all paper-based data will be scanned/entered and saved on secure UniSC servers accessible only to the Chief Investigator and authorised research members. Paper-based data will then be stored in a locked filing cabinet in the Chief Investigators Office, to be accessed only for data checking purposes or at the request of a participant that would like their data permanently removed. All research staff involved in data collection and management who subsequently leave the research study will have revoked access to all physical and/or electronic data. Any recruitment material (e.g. print, media, online) will be stopped/de-activated after completion. Any research staff who subsequently leave following this date must have their access to all physical and electronic data revoked.

8.4. Plans for Sharing and Future Data Use

Data will be used in this project and for other future related projects (Masters and Honours) with primary supervision by members of this research team. If information is shared, all data and results will remain de-identified, and only the data of participants who consented to share their data will be included. Participants will be informed of all planned sharing and future data use in the PICF.

9. Appendices

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