Statistical Analysis Plan (SAP)

Fibre suppLements fOR pre-diAbetes (FLORA)

IRAS Project ID	318937
ClinicalTrials.gov ID	NCT05593926
Protocol version	Version 2.2 (31 Jan 23)
Document status	Version 2.0
Author(s):	Dr Thomas Gurry Dr Caitlin Hall Dr Jan Multmeier
Clinical Investigator	Dr John Luke Twelves
Sponsor	Myota, GmbH

Signature Page

Statistical Analysis Plan for Clinical Study Protocol: FLORA

Fibre suppLements fOR pre-diAbetes (FLORA)

Approved by:

Thomas Gurry, PhD
Chief Scientific Officer & CEO,
Myota Ltd.

Caitlin Hall, PhD
Head of Clinical Research
Myota Ltd.

Jan Multmeier, PhD
Independent Statistician

Document History

Version Number	Author	Date	Change

Glossary of Abbreviations

AE	Adverse Events
BMI	Body Mass Index
BP	Blood Pressure
CI	Chief Investigator
CRP	C-reactive protein
d	Effect size measure, Cohen's *d*
e-Consent	Electronic Consent
EDC	Electronic Data Capture
eTMF	Electronic Trial Master File
FAS	Full Analysis Set
FOS	Fructo-oligosaccharides
GCP	Good Clinical Practice
GOS	Galacto-oligosaccharides
GP	General Practitioner
HbA1c	Glycated haemoglobin
HDL	High-density lipoprotein
ICF	Informed Consent Form
ICH	International Conference on Harmonisation
IL	Interleukin
ISI-OGTT	Insulin Sensitivity Index - Oral Glucose Tolerance Test
ITT	Intention To Treat

LDL	Low-density lipoprotein
	Lindus Health
non-CTIMP	non-Clinical Trial of Investigational Medicinal Product
NSAID	Non-steroidal anti-inflammatory drugs
PHGG	Partially hydrolysed guar gum
PIC	Participant Identification Centre
PIS	Participant Information Sheet
PPI	Patient and Public Involvement
PPS	Per Protocol Analysis Set
REC	Research Ethics Committee
RCT	Randomised Clinical Trial
RN	Research Nurse
SAE	Serious Adverse Event
SAP	Statistical Analysis Plan
SCFAs	Short-Chain Fatty Acids
SOP	Standard Operating Procedure
T2D	Type 2 Diabetes Mellitus
TNF-a	Tumour Necrosis Factor alpha

Table of Contents

Signature Page **2**
Document History **3**
Glossary of Abbreviations **4**
Table of Contents **6**
1.0 Introduction **7**
1.1 Study synopsis 7
1.2 Background and rationale 8
1.2.1 Background 8
1.2.2 Rationale 8
1.3 Study Objectives 9
1.3.1 Primary Objective 9
1.3.2 Secondary Objectives 9
2.0 Study Methods **11**
2.1 Trial design 11
2.2 Randomisation 11
2.3 Sample size 11
2.4 Statistical interim analyses and stopping guidance 11
3.0 Statistical Principles **12**
3.1 Confidence intervals and P values 12
3.2 Adherence and protocol deviations 12
3.3 Analysis populations 12
3.3.1 Intention to Treat (ITT) 12
3.3.2 Per protocol (PP) 12
4.0 Trial Population **12**
4.1 Eligibility 12
4.2 Recruitment 13
4.3 Baseline Measurements 14
4.4 Withdrawal 14
5.5 Analysis **15**
5.1 Outcome definitions 15
5.2 Analysis methods 15
5.3 Missing data 16
5.4 Additional analyses 16
5.5 Sensitivity analyses 16
5.6 Statistical software 16
6.0 References **17**

1.0 Introduction

1.1 Study synopsis

Trial	Parallel randomised controlled trial
Phase	Proof-of-concept
Sample size	60
Study population	Otherwise healthy men and postmenopausal women aged 18-70 with signs of prediabetes, but not clinically diagnosed with diabetes.
Duration	24 weeks
Background	Pre-diabetes is characterised by high blood glucose levels, high plasma cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL). Dietary fibre consumption has been hypothesised to improve these metabolic parameters through microbial fermentation and the subsequent production of short-chain fatty acids (SCFAs).
Objectives and aims	This trial will investigate whether a powdered fibre mix (myota Metabolic Regulator) helps maintain healthy blood glucose levels in participants with prediabetes, where high blood sugar is a risk of diabetes.
Recruitment strategy	Participants will be identified through the Lindus Health Primary Care Network, and social media advertising may also be used. Participants will complete online pre-screening, trial registration and informed consent. Eligibility will be confirmed by the Research Nurse during a video call with the participant.
Randomisation	Participants will be randomised to Placebo or the Myota Metabolic Regulator (powdered fibre mix) with a 1:1 randomisation ratio stratified by gender and BMI.
Intervention	The functional food, Myota Metabolic Regulator, consisting of 20g of a powdered fibre mix, to be taken daily alongside usual diet for 24 weeks.
Comparator Group	Placebo will be 2g of powdered cellulose, to be taken daily alongside usual diet for 24 weeks.
	Participants will be asked to attend an appointment (~2.5-3 hours) at a central clinic in London at baseline, week 16 and week 24. During the appointment, a nurse will obtain blood samples for biomarker measurements (see *Outcomes* for full list), ask the participant to complete an OGTT (baseline and week 16 only) and collect blood pressure recordings. In addition, participant surveys will collect any (Serious) Adverse Events at the end of weeks 4 and 8 and then monthly, usability testing at 16 and 24 weeks, and intervention adherence on a daily basis for 24 weeks. The Lindus Health Research Nurse will contact participants throughout the trial.

1.2 Background and rationale

1.2.1 Background

It is well understood that dietary fibre contributes to a healthy metabolism and maintenance of insulin sensitivity (1). In addition to the more mechanical benefits of fibre, it is fermented by the gut microbiome to produce Short Chain Fatty Acids (SCFAs) which promote healthy metabolic processes (2).

High-throughput multi-omic technologies, genomics, metabolomics and microbiome analyses, are increasingly sensitive and cost-effective tools for gaining mechanistic insights into human health. A particularly fertile terrain for developing novel clinical interventions lies in the human gut microbiota, the composition of which has been associated with a growing number of clinical indications including pre-diabetes. This complex community spans enormous biochemical space to scavenge otherwise indigestible nutrients and other chemicals present in our diet, processing them into substances that can play critical roles in human physiology. It is therefore one of the goals of 21st Century healthcare to design therapies that target and exploit the microbiota and its metabolic output, with particular emphasis on gut diseases such as pre-diabetes which may progress to Type 2 Diabetes and result in significant economic and public health costs.

A critical component to achieving this goal in the context of pre-diabetes is gaining control over microbial fermentation of dietary fibre in the colon, which typically involves the production of the Short Chain Fatty Acids (SCFAs) (2,3). Colonic SCFAs (acetate, propionate and butyrate) have a number of physiological functions, and as such lie at the heart of the symbiotic relationship between host and microbe. It has long been known that microbial butyrate serves as the dominant energy source for colonocytes (4). More importantly in the context of pre-diabetes, butyrate promotes intestinal gluconeogenesis, with beneficial impacts to insulin sensitivity and metabolic status (3). This explains why deficiencies in butyrate production have been associated with Type-2 diabetes and insulin resistance.

This understanding of SCFAs provides us with a more complete picture of the main mechanisms by which dietary fibres act on our metabolism to promote insulin sensitivity to maintain healthy blood glucose in individuals with pre-diabetes and/or Type-2 diabetes (1,5). The first mechanism involves mechanical benefits of more viscous fibres (e.g. arabinoxylan), which reduce post-prandial glucose uptake from a given meal if ingested with the fibre. The second mechanism involves the action of SCFAs on intestinal gluconeogenesis. Finally, it is also understood that SCFAs exert anti-inflammatory pressure on the host's immune system, which is also in line with preventing and/or reversing the pre-diabetic state. These mechanisms are complementary and non-overlapping.

1.2.2 Rationale

The question then arises: can we increase fibre intake to improve SCFA production? Several studies have tested the effect of dietary fibre on plasma SCFA levels, and have shown statistically significant increases in plasma and faecal SCFAs following fibre consumption (6,7,8,9). However, we now know from our research at Myota that different individuals can

sometimes have radically different fermentation capabilities depending on their gut microbiome composition (10). What this means is that when different individuals are fed the same dietary fibre (e.g. inulin) in the same quantity, their microbiomes produce different quantities of SCFAs. These differences in fermentation capability reflect the fact that each individual has a distinct microbiome. They also explain the fact that clinical improvements associated with boosting SCFA production through fibre are significantly diluted by the fact that certain individuals in the respective cohorts may be unable to produce significant quantities of butyrate from the fibre in question. Put differently, we will not observe the full potential efficacy of fibre supplementation unless we account for the fact that different individuals would respond better to different individual fibres as a function of their fermentation capability.

Myota has extensively studied the fermentation capabilities across individuals, and in so doing constructed a database of over 80 volunteers whose fermentation capabilities were analysed experimentally in great detail. This has allowed an understanding of the inter-individual variability in SCFA production from particular fibres, which in turn enabled the calculation of mixtures of fibres that result in the most SCFA (and particularly butyrate) production on average across individuals. Thus, Myota has leveraged cutting edge science in the field of microbiome and nutrition to create a fibre mix which: firstly, includes the relevant quantities of the right fibres in order to obtain the acute (post-prandial) glycaemic benefits of consuming fibre; secondly, uses Myota's proprietary database to compute mixes that promote SCFA production across different individuals with varying microbiome compositions.

In this study, we aim to demonstrate the efficacy of a fibre mix as a functional food in improving clinical biomarkers for people identified as having pre-diabetes, i.e. glycaemic responses (HbA1c, oral glucose tolerance, and fasting blood glucose). Participants will be randomised to either intervention (20g of precision fibre mix daily alongside usual diet) or control (Placebo).

1.3 Study Objectives

1.3.1 Primary Objective

Primary Objective	**Outcome Measure**	**Timepoint(s) of evaluation of this outcome measure**
To compare the effect of Myota's Metabolic Regulator versus placebo on HbA1c levels	Relative change in HbA1c from baseline to 16 weeks	Baseline and 16 weeks

1.3.2 Secondary Objectives

Secondary Objective	**Outcome Measure**	**Timepoint(s) of evaluation of this outcome measure**
To compare the effect of the Myota Metabolic Regulator versus placebo on:		

HbA1c	Change in HbA1c	Baseline, 16 and 24 weeks
Insulin	Change in Insulin	Baseline, 16 and 24 weeks
Insulin sensitivity	Insulin sensitivity, measured using ISI-OGTT	Baseline and 16 weeks
Lipid profile	Cholesterol [total, LDL, HDL] and triglycerides)	Baseline, 16 and 24 weeks
Inflammatory markers	IL-6, IL-8, IL-10, CRP, TNF-a	Baseline, 16 and 24 weeks
Blood pressure	Diastolic and systolic blood pressure	Baseline, 16 and 24 weeks
To investigate the safety of the Myota Metabolic Regulator	Evaluation of overall safety of the Myota Metabolic Regulator by the monitoring of: ● Number of AEs ● Number of SAEs	Continuously for the duration of taking the intervention/placebo
To investigate intervention adherence	Intervention adherence questionnaire	Daily for the duration of the intervention, 24 weeks
To investigate usability	Usability questionnaire	At 16 and 24 weeks

2.0 Study Methods

2.1 Trial design

This trial is a two-arm, individually randomized, single-blind placebo-controlled trial. The total trial duration is 24 weeks. Participants will be assigned to the intervention or placebo for 24 weeks. Primary and secondary outcomes will be assessed at baseline, 16, and 24 weeks (*Section 1.3*).

2.2 Randomisation

Consenting participants who have met all of the eligibility criteria and completed the baseline assessment will be individually randomised into one of the two groups, using a web based system (Sealed Envelope), at a ratio of 1:1, stratified by gender and BMI.

2.3 Sample size

This study aims to recruit 60 participants, men and post-menopausal women aged 18-70 diagnosed with pre-diabetes, but not receiving treatment for type 2 diabetes. As this is a pilot study, our sample size has not been derived statistically, but informed based on previous studies assessing fibre-based interventions in pre-diabetes cohorts. Our sample size is therefore consistent with previous studies assessing fibre-based interventions in similar populations. Results from previous studies (Zhou et al. 2018, n = 43; Peterson et al., 2018, n = 68) suggest that 60 participants is an adequate sample size to capture meaningful differences in our primary and secondary outcome measures. A sample size of 30 in each arm results in a power of 80% to detect effects of at least $d = 0.74$.

2.4 Statistical interim analyses and stopping guidance

One interim analysis will be conducted after 16 weeks of follow-up. Those involved in the day to day conduct of the trial will remain blinded to the outcome of the analysis.

3.0 Statistical Principles

3.1 Confidence intervals and *P* values

Effects will be considered statistically significant if the associated *p*-value is smaller than 0.05.

3.2 Adherence and protocol deviations

Adherence will be reported as a percentage of total intervention days during which participants reported taking the intervention / placebo. This will be assessed by participants completing a daily online survey recording whether they have consumed the full sachet of intervention/placebo. A significant deviation is defined as a participant who consumes the intervention less than 70% of the time.

3.3 Analysis populations

3.3.1 Intention to Treat (ITT)

All randomised study participants. This will be seen as the primary population for the analysis.

3.3.2 Per protocol (PP)

All randomised study participants who complete the trial without significant deviations from the protocol requirements. Analyses of this population is seen as a sensitivity analysis to investigate whether conclusions are sensitive to assumptions regarding the pattern of missing data.

4.0 Trial Population

4.1 Eligibility

Eligibility review will be conducted remotely by the Research Nurse during the telephone/video call with the participant. At the start of the call, the Research Nurse will reiterate the trial procedures and potential risks and benefits associated with taking part, as outlined in the PIS. Informed consent has already been provided online by the participant through signing the ICF, and the nurse will reaffirm consent with the participant during this call. Once reaffirmed, the Research Nurse will use medical history obtained from the participant to confirm whether they are eligible using the inclusion/exclusion criteria checklist. The participant's GP will be asked to confirm any inclusion/exclusion criteria where there is any uncertainty.

For all participants, GPs will be asked to confirm a HbA1c result of between 6.0% and 6.4% in the previous 12 months. A record of screen failures for those who do not meet the inclusion/exclusion criteria will be retained.

Once eligibility has been confirmed, the participant will be booked in to attend their first appointment where baseline measurements will be taken.

4.1.1 Inclusion Criteria

- Able and willing to provide informed consent
- Have a Body Mass Index (BMI) of at least 25 kg/m^2
- Men or post menopausal women aged 18 - 70
- Identified as pre-diabetic (HbA1c 6.0% (42 mmol/mol) to 6.4% (47 mmol/mol)) within the previous 12 months
- Baseline HbA1c result within the range 5.8% (40 mmol/mol) - 6.5% (48 mmol/mol)
- Willing to complete in clinic blood tests and a participant trial survey
- Have access to a smartphone or a computer

4.1.2 Exclusion Criteria

- Receiving medication to treat Type 1 or Type 2 diabetes in the previous 6 months
- Have a Body Mass Index (BMI) <25 kg/m^2 and >45 kg/m^2
- Loss of more than 5% body weight in last 3 months
- Current participation in weight loss program or planned in the next 16 weeks
- Steroid use (except for over the counter NSAID's, topical steroids and inhalers)
- Severe hepatic diseases (including chronic persistent hepatitis, liver cirrhosis or the co-occurrence of positive hepatitis B virus surface antigen and abnormal hepatic transaminase (serum concentrations of alanine transaminase or aspartate transaminase >2.5× the upper normal limit))
- Continuous antibiotic use for >3 days within 4 weeks prior to enrolment
- Continuous use of weight-loss drug for within 3 months of study entry
- Gastrointestinal surgery (except for appendicitis or hernia surgery or co-existing pathology (Crohn's disease, celiac, endometriosis, prostate cancer))
- Severe mental illness within 6 months prior to enrolment
- Receiving drug therapy to treat cholecystitis, peptic ulcers, urinary tract infection, acute pyelonephritis, urocystitis or hyperthyreosis
- Pituitary dysfunction
- Severe organic diseases, including cancer, coronary heart disease, myocardial infarction or cerebral apoplexy
- Infectious diseases, including pulmonary tuberculosis and AIDS
- History of alcoholism or substance abuse
- Significant dyslipidaemia (>3 month use of low dose statins permitted*)
- Severe hypertension (>160/100)
- Using any food supplements for blood glucose control (e.g. chromium picolinate) within 2 months of trial entry

4.2 Recruitment

Participants will be identified through GP practices (Participant Identification Centres - PICs) within the Lindus Health Primary Care Network, and via social media campaigns to facilitate

recruitment. GP practices will be provided with a search for their registers to identify potential participants, who will be invited to take part in the trial via a text message directing them to the trial webpage where they will be able to download a Participant Information Sheet (PIS).

4.3 Baseline Measurements

Participants will attend a central clinic, where the following baseline measurements will be assessed by a qualified nurse:

1. Blood samples for the following biomarkers:
    - HbA1c
    - Insulin
    - Cholesterol ([total, LDL, HDL] and triglycerides)
    - Inflammatory markers (IL-6, IL-8, IL-10, CRP, TNF-a)

2. Insulin Sensitivity Index - Oral Glucose Tolerance Test for Insulin Sensitivity (ISI OGTT)
    - Participants will be asked to fast overnight and after a 10 to 12-hr overnight fast, they will administer a 75-g OGTT. Blood samples will be taken at the following timepoints 0, 30, 60, 90, and 120 min for the measurement of plasma glucose and insulin concentrations.

3. Blood pressure (systolic and diastolic)

Following analysis of the blood samples, only participants with a baseline HbA1c result within the range 5.8% - 6.5% will be considered eligible and subsequently randomised. HbA1c is the average blood glucose level for the last two to three months and so it will naturally vary. Consequently, the range for accepted baseline HbA1c results is slightly broader at baseline, than the initial inclusion criteria.

4.4 Withdrawal

Participants may choose to stop the intervention but may remain on study follow-up. If the participant withdraws completely, all recorded data will be analysed. If the participant starts antidiabetic medications during the trial, they will be withdrawn from the trial as antidiabetic treatment is an exclusion criteria. They will continue to receive follow up surveys to ensure safety monitoring. In addition, the Investigator may discontinue the participation of a participant from the study at any time, if this is considered necessary for any reason including, but not limited to:

- Ineligibility
- Clinical decision for other reasons

The type of withdrawal and reason for withdrawal will be recorded.

5.5 Analysis

5.1 Outcome definitions

5.1.1 Primary Outcome

- HbA1c (%). The percent change between baseline and 16 weeks will be used for analysis.

5.1.2 Secondary Outcomes

- HbA1c (%) between baseline, 16 and 24 weeks
- Glucose (mmol/L) between baseline, 16 and 24 weeks
- Insulin (μU/ml) between baseline, 16 and 24 weeks
- Insulin sensitivity (μU/ml) from ISI-OGTT between baseline and 16 weeks
- Total cholesterol (mmol/L) between baseline, 16 and 24 weeks
- LDL (mmol/L) between baseline, 16 and 24 weeks
- HDL (mmol/L) between baseline, 16 and 24 weeks
- Triglycerides (mmol/L) between baseline, 16 and 24 weeks
- IL-6 (ng/L) between baseline, 16 and 24 weeks
- IL-8 (ng/L) between baseline, 16 and 24 weeks
- IL-10 (ng/L) between baseline, 16 and 24 weeks
- CRP (ng/L) between baseline, 16 and 24 weeks
- TNF-a (ng/L) between baseline, 16 and 24 weeks
- Diastolic blood pressure (mm/Hg) between baseline, 16 and 24 weeks
- Systolic blood pressure (mm/Hg) between baseline, 16 and 24 weeks

5.2 Analysis methods

5.2.1 Descriptive Statistics

All outcome measures will be presented using descriptive statistics. Continuous variables will be summarised using minimum, maximum, mean, standard deviation (SD), median, and interquartile range (IQR). Additionally, the distribution of each variable will be presented using violin and box-plots or other appropriate graphical formats. Binary and categorical variables will be presented using percentages and counts. Descriptive statistics will be presented for each outcome variable at each time point.

For (S)AE, the absolute and relative frequency of each (S)AE will be reported over the whole study duration (24 weeks).

5.2.2 Primary Outcome

A two-tailed t-test will be used to compare the percent change in HbA1c levels from baseline to 16 weeks between treatment and placebo.

5.2.3 Secondary Outcomes

Two-way repeated measures ANCOVA with one within-subject factor (time) and one between-subject factor (condition) will be used to compare HbA1c levels, insulin levels, total cholesterol levels, LDL, HDL and triglyceride levels, inflammatory biomarker levels (including IL-6, IL-8, IL-10, CRP and TNF-a), and diastolic and systolic blood pressure. Post-hoc one-tailed paired t-tests will be performed on significant main or interaction effects to compare differences between groups over time. For non-normally distributed outcome variables, robust ANCOVA and post-hoc comparisons with trimmed means are used. In addition, a two-tailed t-test will be used to compare the change in insulin sensitivity from baseline to 16 weeks between treatment and placebo.

5.3 Missing data

Primary and secondary analyses will be performed on the ITT population. Outcome data will be imputed using multiple imputation (11).

5.4 Additional analyses

Exploratory analyses will examine interdependencies of the primary and secondary outcomes, and sub-group analyses including the effects of baseline HbA1c, insulin sensitivity, fasting glucose, sex and age groups on the percent change in HbA1c level.

5.5 Sensitivity analyses

As a sensitivity analysis, the primary and secondary outcomes will be analyzed using the PP population to investigate whether conclusions are sensitive to assumptions regarding the pattern of missing data.

5.6 Statistical software

Python will be used for all statistical analysis.

6.0 References

1. Reynolds AN, Akerman AP, Mann J (2020). Dietary fibre and whole grains in diabetes management: Systematic review and meta- analyses. PLoS Med 17(3): e1003053.
2. Zhao L, Zhang F, Ding X, Wu G, Lam YY, Wang X, ... Zhang C. (2018). Gut bacteria selectively promoted by dietary fibers alleviate type 2 diabetes. Science, 359(6380), 1151-1156.
3. De Vadder F, Kovatcheva-Datchary P, Goncalves D, Vinera J, Zitoun C, Duchampt A, Bäckhed F, Mithieux G. Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits. Cell. 2014 Jan 16;156(1-2):84-96.
4. Roediger WEW. Utilization of nutrients by isolated epithelial cells of the rat colon. Gastroenterology 1982;83:424–9.
5. Peterson CM, Beyl RA, Marlatt KL, Martin CK, Aryana KJ, Marco ML, Martin RJ, Keenan MJ, Ravussin E. Effect of 12 wk of resistant starch supplementation on cardiometabolic risk factors in adults with pre-diabetes: a randomized controlled trial. Am J Clin Nutr. 2018 Sep 1;108(3):492-501.
6. Gurry T, & Scapozza L (2020). Exploiting the gut microbiota's fermentation capabilities towards disease prevention. Journal of Pharmaceutical and Biomedical Analysis, 189, 113469.
7. Yu X, Gurry T, Nguyen L, Richardson HS, & Alm, EJ (2020). Prebiotics and community composition influence gas production of the human gut microbiota. MBio, 11(5), e00217-20.
8. Bai J, Li Y, Zhang W, Fan M, Qian H, Zhang H,... Wang L (2021). Effects of cereal fibers on short-chain fatty acids in healthy subjects and patients: a meta-analysis of randomized clinical trials. Food & Function, 12(15), 7040-7053.
9. Velázquez M, Davies C, Marett R, Slavin JL, & Feirtag JM (2000). Effect of oligosaccharides and fibre substitutes on short-chain fatty acid production by human faecal microflora. Anaerobe, 6(2), 87-92.
10. Gurry T, Nguyen LTT, Yu X, & Alm EJ (2021). Functional heterogeneity in the fermentation capabilities of the healthy human gut microbiota. Plos one, 16(7), e0254004.
11. Carpenter, J. R. , Roger, J. H. , & Kenward, M. G. (2013). Analysis of longitudinal trials with protocol deviation: A framework for relevant, accessible assumptions, and inference via multiple imputation. Journal of Biopharmaceutical Statistics, 23(3), 1352–1371.