Statistical Analyses Plan

Administrative information

Study Title:	Effect of dairy products on insulin resistance in overweight and obese individuals with prediabetes
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Revision History:

Protocol Version	SAP Version Number	SAP Version Date	Section Changed	Summary of Changes	Date of revision

Signature page

Roles and responsibilities

Name	Role	Institution	Signature
Sergio Burgos, PhD	Principal investigator	McGill University	
Roger Cue, PhD	Trial statistician	McGill University	
Yi Jin, RD	Graduate student	McGill University	

Contributions

Sergio Burgos and Yi Jin drafted the initial version of the statistical analyses plan (SAP) based on the study protocol.
Roger Cue reviewed the SAP and provided critical feedback.

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ABBREVIATION AND DEFINITIONS

ANCOVA	Analysis of covariance
ASA24	Automated self-administered 24-hour dietary assessment tool
BMI	Body mass index
CI	Confidence interval
CONSORT	Consolidated Standards of Reporting Trials
CRP	C-reactive protein
ELISA	Enzyme-linked immunosorbent assay
HDL-C	High-density lipoprotein-cholesterol
HOMA-IR	Homeostatic model assessment of insulin resistance
IGT	Impaired glucose tolerance
IL-6	Interleukin-6
IPAQ	International physical activity questionnaire
IQR	Interquartile range
LDL-C	Low-density lipoprotein-cholesterol
MET	Metabolic equivalent of task
MUHC	McGill University Health Centre
Non-HDL-C	Non-high-density lipoprotein cholesterol
OGTT	Oral glucose tolerance test
RCT	Randomized controlled trial
REDCap	Research Electronic Data Capture
SAP	Statistical analyses plan
SD	Standard deviation
T2D	Type 2 diabetes
TC	Total cholesterol
TG	Triglyceride
TNF-α	Tumor necrosis factor-alpha

1 INTRODUCTION

Epidemiological studies have shown a reduced risk of type 2 diabetes (T2D) with higher total dairy intake, particularly low-fat and fermented dairy products [1-7]. This association could be mediated by dairy fat enhancing insulin sensitivity based on the strong inverse association between specific dairy-derived plasma fatty acid biomarkers of dairy fat intake with T2D incidence in prospective studies [8-13], also positively associated with increased insulin sensitivity in cross-sectional studies [11, 14, 15]. Insulin resistance is an established risk factor for T2D that precedes its development and is potentially reversible through dietary interventions. Results from published randomized controlled trials (RCT) are inconsistent regarding the effect of total dairy intake on insulin sensitivity [16-23]. This stems partly from design issues relating to study population selection, intervention duration, primary outcome definition, and endpoint assessment methods, which lack the requisite specificity and sensitivity to test for the effect. Therefore, we will conduct an RCT to determine the effects of dairy (≤1 serving/day versus 2-3 servings/day) and dairy fat content (reduced versus regular fat) on insulin sensitivity – a major risk factor for T2D and cardiovascular disease – as the primary outcome and other cardiometabolic risk factors as secondary outcomes. In addition, we will validate existing and identify new lipid biomarkers of dairy fat intake that could be used further to clarify the association between dairy intake and T2D.

Research hypotheses

Primary hypothesis

Null Hypothesis (H_0):
There is no significant difference in the change in whole-body insulin sensitivity (measured during a hyperinsulinemic-euglycemic clamp) between participants consuming a limited amount of dairy (≤1 serving/day) and those consuming 2 to 3 servings/day (reduced or regular fat) over a 12-week intervention period.

Alternative Hypothesis (H_1):
Participants consuming 2 to 3 servings/day of dairy (reduced or regular fat) will exhibit a significant change in whole-body insulin sensitivity compared to those consuming a limited amount of dairy (≤1 serving/day).

Secondary hypothesis

Null Hypothesis (H_0):
There is no significant difference in the change in whole-body insulin sensitivity (measured during a hyperinsulinemic-euglycemic clamp) between participants consuming 2 to 3 servings/day of reduced-fat dairy and those consuming 2 to 3 servings/day of regular-fat dairy over a 12-week intervention period.

Alternative Hypothesis (H_1):
Participants consuming regular-fat dairy will exhibit a significant change in whole-body insulin sensitivity compared to those consuming reduced-fat dairy.

Study objectives

The primary objective is to determine the effects of dairy and dairy fat consumption on the change in whole-body insulin sensitivity as measured by a hyperinsulinemic-euglycemic clamp before and after a 12-week dairy intervention.

The secondary objective is to determine the effects of dairy and dairy fat consumption on glycemic measures, body composition, and other cardiometabolic risk factors.

2 STUDY METHODS

2.1 Trial design

We will conduct a 12-week three-arm parallel-group RCT in females and males aged 30-65 years with overweight or obesity with prediabetes. The intervention will be preceded by a 2-week run-in period in which participants consume one serving/day of reduced-fat milk, cheese, or yogurt to promote continuation during the trial. Participants will then be randomly assigned to one of three groups in a 1:1:1 allocation ratio stratified by sex.

Groups

1. Limited dairy: Participants will consume ≤1 dairy serving/day
2. Reduced-fat dairy: Participants will consume 2-3 servings/day of skim milk, fat-free yogurt, and reduced-fat cheese.
3. Regular-fat dairy: Participants will consume 2-3 servings/day of regular-fat milk, yogurt, and cheese.

Randomization

We will use stratified randomization to ensure a similar sex ratio across all participants. A consulting study statistician will stratify participants using a computer-generated randomization table and enter the randomization module in Research Electronic Data Capture (REDCap). Details of the randomization method will be held securely within the statistics master file.

Blinding

Due to the nature of the test products, participants cannot be blinded to the diet intervention. The research dietitian and clinical research coordinator, who need to know the treatment assignment for counselling and to hand the test products to the participants, will not be blinded to treatment. The outcome assessors will be blinded to treatment allocation.

2.2 Study procedures

The study comprises seven in-person visits: a screening visit, a run-in visit (week -2), two in-patient visits: one at baseline (week 0) and the second 12 weeks into the intervention (week 12), two out-patient visits (week 4 and 8), and a follow-up visit (week 13). Table 1 outlines the procedures performed at each study visit.

Table 1. Schedule of procedures

Timing	Visit 1 ~Week -5 Screening	Visit 2 Week -2 Run-in	Visit 3 Week 0 Baseline	Visit 4 Week 4 Intervention	Visit 5 Week 8 Intervention	Visit 6 Week 12 Intervention	Visit 7 Week 13 Follow-up
Informed consent	X						
Height	X						
Weight	X	X	X	X	X	X	X
Waist circumference	X		X	X	X	X	X
Blood pressure	X		X	X	X	X	X
Questionnaires							
Demographics	X						
Family history	X						
Medical history	X						
International Physical Activity Questionnaire	X		X	X	X	X	X
24-hour food recall	X						X
Dairy food frequency questionnaire	X						
Fasting blood sample							
Glucose	X		X			X	X
Insulin	X		X			X	X
Glycated hemoglobin	X						X
C-reactive protein			X			X	
Lipids	X		X			X	
Dairy biomarkers	X		X	X	X	X	
Oral glucose tolerance test	X						X
Dual-energy X-ray absorptiometry			X			X	
Hyperinsulinemic-euglycemic clamp			X			X	
Indirect calorimetry			X			X	
Fat biopsy			X			X	
Dairy intake log			X	X	X	X	X
3-day food diaries			X	X	X	X	
Accelerometry			X			X	
Chest X-ray	X						
Electrocardiogram	X						
Physical exam	X						**If needed**

2.3 Outcomes

Primary outcome

- **The change in whole-body insulin sensitivity**, defined by the glucose infusion rate during the steady state of a hyperinsulinemic-euglycemic clamp, adjusted for lean body mass and serum insulin concentration during the same period [24]. The outcome will be assessed as the difference between week 0 and week 12.

Secondary outcomes

Glycemic measures:

- Change in fasting plasma glucose concentrations [time frame: difference between screening and 13-week follow-up visit] as measured by glucose oxidase analyzer (Analox).
- Change in fasting serum insulin concentrations [time frame: difference between screening and 13-week follow-up visit] as measured by enzyme-linked immunosorbent assay (ELISA).
- Change in homeostatic model assessment of insulin resistance (HOMA-IR) [time frame: difference between screening and 13-week follow-up visit] as calculated from fasting glucose and insulin concentrations [25].
- Change in glucose tolerance [time frame: difference between screening and 13-week follow-up visit] as measured by plasma glucose concentration at 2-hour of an oral glucose tolerance test (OGTT).
- Change in Matsuda index [time frame: difference between screening and 13-week follow-up visit] as calculated from plasma glucose and insulin concentrations during an OGTT [26].
- Change in insulinogenic index [time frame: difference between screening and 13-week follow-up visit] as calculated from the ratio of serum insulin concentration and plasma glucose at 30 min minus 0 min during an OGTT [27].
- Change in oral disposition index [time frame: difference between screening and 13-week follow-up visit] as calculated from the product of the Matsuda index and insulinogenic index [26].
- Change in fasting serum glycated hemoglobin percent [time frame: difference between screening and 13-week follow-up visit] as measured by the MUHC Central Laboratories.

Body composition:

- Change in total lean body mass [time frame: difference between baseline and 12-week intervention visit] as measured by dual-energy X-ray absorptiometry (DXA).
- Change in total fat mass [time frame: difference between baseline and 12-week intervention visit] as measured by DXA.
- Change in visceral fat mass [time frame: difference between baseline and 12-week intervention visit] as estimated by DXA [28].

Cardiovascular risk factors:

- Change in systolic blood pressure [time frame: difference between baseline and 12-week intervention visit] as measured by automated blood pressure monitor.

- Change in diastolic blood pressure [time frame: difference between baseline and 12-week intervention visit] as measured by automated blood pressure monitor.
- Change in fasting serum total cholesterol (TC) concentration [time frame: difference between baseline and 12-week intervention visit] as measured by the MUHC Central Laboratories.
- Change in fasting serum total triglycerides (TG) concentration [time frame: difference between baseline and 12-week intervention visit] as measured by the MUHC Central Laboratories.
- Change in fasting serum total high-density lipoprotein-cholesterol (HDL-C) [time frame: difference between baseline and 12-week intervention visit] as measured by the MUHC Central Laboratories.
- Change in fasting total low-density lipoprotein-cholesterol (LDL-C) [time frame: difference between baseline and 12-week intervention visit] as calculated from fasting serum TC, HDL-C, and TG [29].
- Change in fasting total non–high-density lipoprotein cholesterol (non-HDL-C) [time frame: difference between baseline and 12-week intervention visit] as calculated from fasting serum TC and HDL-C.
- Change in fasting serum C-reactive protein (CRP) [time frame: difference between baseline and 12-week intervention visit] as measured by the MUHC Central Laboratories.
- Change in fasting serum interleukin-6 (IL-6) [time frame: difference between baseline and 12-week intervention visit] as measured by ELISA.
- Change in fasting serum tumor necrosis factor-alpha (TNF-α) [time frame: difference between baseline and 12-week intervention visit] as measured by ELISA.

Other outcome measures

- Change in weight and body mass index (BMI) [time frame: difference between baseline and 12-week intervention visit] as calculated from weight and height.
- Change in resting energy expenditure [time frame: difference between baseline and 12-week intervention visit] as measured by indirect calorimetry.
- Change in metabolic flexibility [time frame: difference between baseline and 12-week intervention visit] as measured by the difference in respiratory quotient between basal and hyperinsulinemic periods of a hyperinsulinemic-euglycemic clamp [30].
- Change in physical activity energy expenditure [time frame: difference between baseline and 12-week intervention visit] as measured by accelerometry.
- Change in total energy expenditure [time frame: difference between baseline and 12-week intervention visit] as estimated from resting energy expenditure and physical activity energy expenditure [31].
- Change in total energy intake [time frame: difference between baseline and 12-week intervention visit] based on 3-day food records and analyzed by Keenoa® [32].
- Averaged daily macronutrient and micronutrient intakes [time frame: difference between baseline and 12-week intervention visit] based on 3-day food records and analyzed by Keenoa® [32].

- Averaged daily food group intakes [time frame: difference between baseline and 12-week intervention visit] based on 3-day food records and analyzed by Keenoa® [32].
- Change in metabolic equivalent of task (MET) [time frame: difference between baseline and 12-week intervention visit] as estimated by the International Physical Activity Questionnaire (IPAQ) – Short Form [33].
- Change in sitting time [time frame: difference between baseline and 12-week intervention visit] as estimated by the IPAQ – Short Form [33].
- Change in MET [time frame: difference between baseline and 12-week intervention visit] as measured by accelerometry.
- Change in the percentage of time spent in sedentary, light, moderate and vigorous activity [time frame: difference between baseline and 12-week intervention visit] as measured by accelerometry.

2.4 Study adherence

We will assess adherence to the dietary intervention at each visit based on dairy intake logs, where participants will record the amount and types of dairy foods they consume daily. Participants will also be asked to complete one 3-day food diary (including one weekend day) every month using a food tracking mobile application (Keenoa®) [32] or hand-written paper copies. The two types of records will be considered equivalent and used to assess total energy intake.

Adherence will be assessed based on:

- Averaged daily intake of study dairy foods during the run-in period and the intervention period as calculated from dairy intake logs.
- Averaged daily intake of non-study dairy foods during the run-in period and the intervention period based on dairy intake logs and analyzed by Automated Self-Administered 24-Hour (ASA24®) Dietary Assessment Tool [34].
- Change in the proportions of 15:0, 17:0 and/or t16:1n7 fatty acids in serum lipid fractions [time frame: difference between baseline and 12-week intervention visit] as measured by gas chromatography mass spectrometry.

2.5 Data management

All study-related clinical data will be collected and audited using REDCap™ hosted at MUHC. Dietary data will be collected with ASA24® for 24-hour dietary recalls and with Keenoa® for 3-day dietary records. Objective physical activity data will be collected using Actigraph® wGT3X-BT and processed using ActiLife® (version 6.13.4) [35].

2.6 Timing of final analysis

The trial will finish with the last follow-up appointment. The data will be cleaned, verified, and locked. The statistical analysis will commence once the principal investigator confirms the final database lock.

3 STATISTICAL ANALYSES

All statistical analyses will be performed blinded using R (version 4.4.1 2024-06-14) and RStudio (version 2024.09.0+375).

3.1 Descriptive Statistics

Baseline characteristics will be summarized across treatment groups. Continuous variables will be presented as means and standard deviations (SD) or medians and interquartile ranges (IQR), depending on their distribution (assessed using the Shapiro-Wilk test). Categorical variables will be summarized using frequencies and percentages.

3.2 Primary Analyses

The effects of the interventions on all measured outcomes will first be assessed in an intent-to-treat analysis, which will include all randomized participants. In addition, a per-protocol analysis, including only the participants who adhere to the study protocol, will be performed. Results of intent-to-treat and per-protocol analyses will be interpreted together.

Analysis of covariance (ANCOVA) will be employed to assess the treatment effects (limited dairy, reduced-fat dairy, and regular-fat dairy) on changes in all measured outcomes following the dairy intervention. The model will be adjusted for sex (male and female), baseline outcome values (continuous), age (continuous), and BMI (continuous). Predicted values and residuals will assess model adequacy. If model assumptions are violated, pre- and post-intervention outcome values will be log-transformed to compute the change, with back-transformation applied to ensure interpretability.
Specific hypotheses will be tested using the following preplanned orthogonal contrasts:

1. the overall effect of dairy intake (limited dairy vs. the average of reduced-fat and regular-fat dairy, contrast: [-2, 1, 1]), and
2. the effect of dairy fat intake (reduced-fat vs. regular-fat dairy, contrast: [0, 1, -1]).

Because the two contrasts are pre-planned and independent from each other, no adjustment for multiple comparisons is needed. A two-sided $p < 0.05$ for each contrast will be considered statistically significant. No correction for multiple testing will be performed for secondary and other outcomes as these outcomes are exploratory.

Estimated mean change and 95% confidence interval (CI) for each treatment group will be used to determine the within-group difference before and after the intervention. Changes will be considered significant if the 95% CI does not include 0.

To handle missing data, we will apply multiple imputations by chained equations, assuming that data are missing at random. The imputation model will include all relevant covariates (baseline values and demographic factors) to predict missingness. Twenty to thirty imputed datasets, depending on the level of missingness, will be generated [36, 37] and results will be pooled using Rubin's rules.

3.3 Secondary Analyses

Instead of using the two pre-planned contrasts, the same ANCOVA model in the primary analyses will be used to perform a global F-test. If the global test is significant ($p < 0.05$), pairwise comparisons will be performed to identify group differences and adjusted using Tukey's Honest Significant Difference test and discussed as exploratory analyses.

3.4 Mediation analysis

To explore potential causal mechanisms, we will conduct a mediation analysis to determine whether changes in inflammatory markers (CRP, IL-6, TNF-a) and body composition measures (total lean, total fat, and visceral fat mass) explain the effects of dairy consumption on insulin sensitivity.

3.5 Sensitivity analyses

Sensitivity analyses will be compared to the primary analysis to test the robustness of the primary findings.

- A complete case analysis will be performed, where missing data will not be imputed.
- Outliers will be identified and excluded as values >2 SD from the mean or using Tukey's method (1.5×IQR). If the results differ with and without the outliers, we will apply 90% Winsorization to minimize the influence of extreme values.

3.6 Additional exploratory analyses as applicable

- Impact of sex: A treatment by sex interaction term will be added to the primary model to assess whether the treatment effects differ by sex.
- Impact of prediabetes phenotypes: The prediabetes phenotype (presence vs. absence of impaired glucose tolerance IGT) will be added as a fixed factor to the primary model, along with its interaction with treatment group to assess whether the treatment effects differ by the prediabetes phenotype.
- Impact of different types of dairy foods: Servings of each dairy product (milk, yogurt and cheese) consumed will be added as covariates to the primary model to assess their independent effects on outcomes and to account for variability in intake.
- Impact of lifestyle changes: Changes in physical activity (measured as changes in METs from accelerometry) and dietary intake (changes in macronutrient and micronutrient intakes from 3-day food records) will be added as covariates to the primary model to control for their potentially confounding effects on the outcomes.

4 TABLES AND FIGURES DRAFT

Table 1. Baseline characteristics

	Total (n = 60)	Limited dairy (n = 20)	Reduced-fat dairy (n = 20)	Regular-fat dairy (n = 20)
General				
Age (years)				
Sex, male/female (n)				
Body anthropometry and composition				
Body weight (kg)				
BMI (kg/m^2)				
Waist circumference (cm)				
Male				
Female				
Lean body mass (kg)				
Fat mass (kg)				
Visceral fat mass (g)				
Glycemic measures				
Fasting glucose (mmol/L)				
Glucose 2h post-OGTT (mmol/L)				
Glycated hemoglobin (%)				
Prediabetes phenotype				
Presence of IGT (%)				
Absence of IGT (%)				
Clamp-measured insulin sensitivity				
Insulin sensitivity indices				
HOMA-IR				
Matsuda index				
β-cell function indices				
Insulinogenic index				
Oral disposition index				
Cardiovascular risk factors				
Systolic blood pressure (mmHg)				
Diastolic blood pressure (mmHg)				
TC (mmol/L)				
TG (mmol/L)				
HDL-C (mmol/L)				
LDL-C (mmol/L)				
Non-HDL-C (mmol/L)				
CRP (mg/L)				
IL-6 (pg/mL)				
TNF-α (pg/mL)				

Table 2. Within-group changes (0-12 weeks) in other cardiometabolic outcomes

	Limited dairy	Reduced-fat dairy	Regular-fat dairy	P_{dairy}	$P_{dairy\ fat}$
Body anthropometry and composition					
Body weight (kg)					
BMI (kg/m^2)					
Lean body mass (kg)					
Fat mass (kg)					
Visceral fat mass (g)					
Blood pressure					
Systolic (mmHg)					
Diastolic (mmHg)					
Lipid profile					
TC (mmol/L)					
TG (mmol/L)					
HDL-C (mmol/L)					
LDL-C (mmol/L)					
Non-HDL-C (mmol/L)					
Inflammatory markers					
CRP (mg/L)					
IL-6 (pg/mL)					
TNF-α (pg/mL)					
Energy metabolism					
Resting energy expenditure					
Δ Respiratory quotient					

Figure 1. Consolidated Standards of Reporting Trials (CONSORT) participant flow diagram.

Figure 2. Clamp-measured whole-body insulin sensitivity at baseline and 12 weeks into the intervention and change by group

Figure 3. Changes in glycemic measures, insulin sensitivity indices, and β-cell function indices during the intervention period by group
(A) fasting glucose, (B) fasting insulin, (C) glycated hemoglobin, (D) glucose tolerance, (E) HOMA-IR, (F) Matsuda index, (G) insulinogenic index; (H) oral deposition index.

Supplementary materials

Supplementary Table 1. Participant baseline demographic information

	Total (n = 60)	Limited dairy (n = 20)	Reduced-fat dairy (n = 20)	Regular-fat dairy (n = 20)
Ethnicity (%)				
Asian				
Black or African American				
Hispanic				
White				
Other				
Education (%)				
High school diploma				
College diploma				
University degree				
Other				
Household income (%)				
<$15,000				
$15,000 – $39,000				
$40,000 – $79,999				
$80,000 – $124,999				
>$125,000				
Prefer not to answer/Don't know				

Supplementary Table 2. Adverse events after randomization

	Total, n (%)	Limited dairy, n (%)	Reduced-fat dairy, n (%)	Regular-fat dairy, n (%)
Participants with ≥ 1 adverse event				
Gastrointestinal discomfort				
Bloating				
Diarrhea				
Other				
Complications associated with experimental procedures				
Excessive bleeding from muscle biopsy				
Pain from muscle biopsy				
Felt uncomfortable after hyperinsulinemic-euglycemic clamp				
Felt uncomfortable during indirect calorimetry				

Gastrointestinal discomfort includes events that are likely and unlikely related to the dairy intervention.

Supplementary Table 3. Adherence to the run-in period and the dairy intervention period

	Limited dairy		Reduced-fat dairy		Regular-fat dairy	
	Run-in	Intervention	Run-in	Intervention	Run-in	Intervention
Intake of study dairy foods (dairy intake logs)						
Reduced-fat dairy						
Reduced-fat milk						
Reduced-fat yogurt						
Reduced-fat cheese						
Regular-fat dairy						
Regular-fat milk						
Regular-fat yogurt						
Regular-fat cheese						
Intake of non-study dairy foods (dairy intake logs)						
Non-study dairy						
Serum biomarkers of dairy fat intake						
15:0 (% of total)						
17:0 (% of total)						
*t*16:1n-7 (% of total)						

Dairy intakes are expressed as serving/day.
One serving equals 250 mL milk, 175 g yogurt, or 50 g cheese.

Supplementary Table 4. Physical activity at baseline and 12 weeks into the intervention

	Limited dairy			Reduced-fat dairy			Regular-fat dairy		
	Week 0	Week 12	Δ	Week 0	Week 12	Δ	Week 0	Week 12	Δ
IPAQ									
MET									
Sitting time (hour)									
Accelerometry									
MET									
Sedentary activity (%)									
Light activity (%)									
Moderate activity (%)									
Vigorous activity (%)									
Physical activity energy expenditure (kcal)									
Total energy expenditure (kcal/day)									

Supplementary Table 5. Dietary intake at baseline and 12 weeks into the intervention

	Limited dairy			Reduced-fat dairy			Regular-fat dairy		
	Week 0	Week 12	Δ	Week 0	Week 12	Δ	Week 0	Week 12	Δ
Total energy intake (kcal)									
Macronutrients									
Fat (%)									
SFA (g)									
MUFA (g)									
PUFA (g)									
TFA (g)									
Carbohydrate (%)									
Sugar (g)									
Fiber (g)									
Protein (%)									
Micronutrients									
Sodium (mg)									
Potassium (mg)									
Magnesium (mg)									
Iron (mg)									
Calcium (mg)									
Vitamin D (IU)									
Food groups									
Fruit (cup eq.)									
Vegetables (cup eq.)									
Total grains (oz. eq.)									
Whole grains (oz. eq.)									
Total meat (oz. eq.)									
Soy, nuts and legumes (oz. eq.)									
Total dairy (cup eq.)									

SFA saturated fatty acid, MUFA mono-unsaturated fatty acid, PUFA poly-unsaturated fatty acid, TFA trans fatty acids, IU international unit, cup eq. cup equivalent, oz. eq. ounce equivalent

Supplementary Figure 1. Visual abstract

Supplementary Figure 2. Study design

Supplementary Figure 3. Effect of the dairy interventions (A, limited dairy; B, reduced-fat dairy; C, regular-fat dairy) on glucose infusion rate across a 2-hour hyperinsulinemic-euglycemic clamp

Supplementary Figure 4. Directed acyclic graph of the causal effect of dairy intake on the change in whole-body insulin sensitivity through changes in serum inflammatory markers and body composition measures

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