Statistical analysis plan (SAP)

Liver Fat as a Dietary Target for Treating Cardiometabolic Disorders in Prediabetes and Type 2 Diabetes: a Randomized Study (**NAFLDiet**)

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Abbreviations

ApoA1	Apolipoprotein A1
ApoB	Apolipoprotein B
BIA	Bioelectrical impedance analysis
BMI	Body mass index
CRP	C-reactive protein
CVD	Cardiovascular disease
DNL	De-novo lipogenesis
FMD	Flow-mediated dilation
GC	Gas chromatography
GLM	General Linear Model
HbA1c	Glycated hemoglobin
HDL	High-density lipoprotein
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
IQR	Interquartile range
ITT	Intention to treat
LDL	Low-density lipoprotein
MRI	Magnetic resonance imaging
NAFLD	Non-alcoholic fatty liver disease
OGTT	Oral glucose tolerance test
PNPLA3	Patatin-like phospholipase domain protein 3
PP	Per protocol
PUFA	Polyunsaturated fatty acid(s)
PWV	Pulse-wave velocity
SCD-1	Stearoyl coenzyme-A desaturase 1
SD	Standard deviation
T2D	Type 2 diabetes
UPLC-MS/MS	Ultra-high performance liquid chromatography coupled to tandem mass spectrometry

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1. Introduction

Randomized controlled studies investigating the impact of replacing dietary carbohydrates with polyunsaturated fat (PUFA) on liver fat content and cardiometabolic risk in individuals with prediabetes and T2D are lacking. Also, the effects of a Healthy Nordic Diet on liver fat content and glycemic control have not be investigated. This study therefore aims to:

- Investigate the effects of the diets on liver fat content (primary aim)
- Investigate the effects of the diets on pancreatic fat, visceral fat, lean tissue, glycemic and lipid control
- Investigate the effects of the diets on plasma markers of de novo lipogenesis (DNL) and desaturation (i.e. stearoyl-Coenzyme desaturase 1, SCD-1) as well as on hepatic DNL using MRI spectroscopy
- Investigate gene-diet interactions, especially if common gene variants (e.g. in PNPLA3) known to increase liver fat and dyslipidemia, may modify the dietary effects.
- Perform lipidomic analyses to identify potential mechanistic pathways that may associate with diet-induced changes in liver fat, pancreatic fat, visceral fat, insulin sensitivity, dyslipidemia or DNL

Our hypothesis is that a customized diet will effectively reduce liver fat through suppression of hepatic DNL and SCD-1 activity, and thereby improve atherogenic dyslipidemia, insulin resistance and hyperglycemia in individuals with prediabetes and T2D.

2. Study design

Study Type: Interventional (Single-center Clinical Trial)

Estimated Enrollment: 150 participants

Allocation: Randomized

Intervention Model: Parallel assignment

Intervention Model Description: Parallel assignment

Masking: Double (Care Provider, Outcomes Assessors)

Primary Purpose: Treatment

Hypothesis Testing Framework: Superiority Trial

Eligibility criteria are presented below:

Inclusion criteria

- Men and women
- 30-75 years
- BMI 25-40
- T2D (duration ≤10 years, no insulin treatment) or prediabetes (ADA definition 2019) without diagnosed cardiovascular disease (CVD) during the last 2 years (e.g. myocardial infarction, stroke or angina pectoris)

Exclusion criteria

- BMI >40
- Alcohol intake >20 g/day
- Unwillingness to follow a new prescribed diet for 1 year
- Diet-induced weight loss (≥10%) the preceding 3 months of screening
- Malignant disease
- Severe kidney and liver disease
- Heart failure or other severe CVD
- Claustrophobia or metal parts in the body (MRI)

Subjects enrolled in this study will be allocated to three different diet groups in a 1:1:1 allocation ratio using stratified randomization with type-2 diabetes status (yes/no) and gender (male/female) as stratifying factors. Subjects will be followed prospectively for 12 months.

The three diet-groups are:

Experimental: Customized diet to reduce liver fat
Ad libitum diet high in plant-derived PUFA and lower in carbohydrates

Experimental: Healthy Nordic diet
Ad libitum diet, based on Nordic foods, higher in carbohydrates (high fiber/low GI) and lower in fat but rich in monounsaturated fatty acids (MUFA) and PUFA

Active Comparator: Control
Ad libitum diet in accordance with the Nordic Nutrition Recommendations

Further details of each diet can be found in the study protocol registered at ClinicalTrials.gov (NCT04527965).

2.1. Sample size calculation

The sample size calculation was based on Lehr´s formula for comparison between two groups, assuming equal treatment effects for the two experimental diets. The standard deviation (SD) of the change in liver fat was 1.95 and 2.42 in two of our previous trials (1,2). As the population in the NAFLDiet trial will differ (e.g. higher prevalence of type 2 diabetes) compared to these other trials, we assume a SD of 3 for the current calculation. Given that 5% liver fat is the cut-off for NAFLD, we considered a 2% difference between groups as clinically relevant. To detect a difference of 2%-points in liver fat between the control group and one experimental group, n=36 individuals per group are needed with β=0.80 and α=0.05. To allow for 25% drop-out, n=50 individuals per group will be included. Thus, a total of n=150 individuals will be randomized.

3. Aims and objectives

The overall aim of this study is to investigate the long-term impact of a customized diet aimed at reducing liver fat specifically and a healthy Nordic diet on ectopic fat (liver, pancreatic and visceral) and cardiometabolic risk in individuals with prediabetes and type 2 diabetes.

4. Outcomes

4.1. Primary outcome

- Between-group changes in liver fat content between baseline and month 12

  [Time Frame: 12 months] [Unit: %] [Variable: Continuous]

  Assessed by magnetic resonance imaging (MRI)

4.2. Secondary outcomes

- Between-group changes in visceral adipose tissue mass between baseline and month 12

  [Time Frame: 12 months] [Unit: L] [Variable: Continuous]

  Assessed by magnetic resonance imaging (MRI)

- Between-group changes in lean tissue mass between baseline and month 12

  [Time Frame: 12 months] [Unit: L] [Variable: Continuous]

  Assessed by magnetic resonance imaging (MRI)

- Between-group changes in total body fat mass between baseline and month 12

  [Time Frame: 12 months] [Unit: L] [Variable: Continuous]

  Assessed by magnetic resonance imaging (MRI)

- Between-group changes in body weight between baseline and month 12

  [Time Frame: 12 months] [Unit: kg] [Variable: Continuous]

  Assessed by using a Tanita bioelectrical impedance analysis (BIA) scale

- Between-group changes in glycated hemoglobin (HbA1c) between baseline and month 12

  [Time Frame: 12 months] [Unit: mmol/mol] [Variable: Continuous]

  Assessed by routine clinical chemistry

- Between-group changes in Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) between baseline and month 12

  [Time Frame: 12 months] [Unit: none] [Variable: Continuous]

  Calculated by multiplying fasting plasma glucose (mmol/L) with fasting serum insulin (mU/L) and dividing the product by 22.5.

  Assessed by routine clinical chemistry

- Between-group changes in fasting plasma glucose between baseline and month 12

  [Time Frame: 12 months] [Unit: mmol/L] [Variable: Continuous]

  Assessed by routine clinical chemistry

- Between-group changes in fasting serum insulin between baseline and month 12

  [Time Frame: 12 months] [Unit: mU/L] [Variable: Continuous]

Assessed by routine clinical chemistry

- Between-group changes in systolic blood pressure between baseline and month 12

  [Time Frame: 12 months] [Unit: mmHg] [Variable: Continuous]

  Assessed by using an automated blood pressure monitor

- Between-group changes in diastolic blood pressure between baseline and month 12

  [Time Frame: 12 months] [Unit: mmHg] [Variable: Continuous]

  Assessed by using an automated blood pressure monitor

- Between-group changes in plasma lipids (total cholesterol, LDL cholesterol, triglycerides, HDL cholesterol, apoB and apoA1) between baseline and month 12

  [Time Frame: 12 months] [Unit: mmol/L or g/L (apoB and apoA1)] [Variable: Continuous]

  Assessed by routine clinical chemistry

- Between-group changes in circulating inflammatory markers (CRP, Tumor Necrosis Factor Alpha-receptor 1 and 2, Interleukin-1 receptor antagonist, Fibroblast growth factor 21) between baseline and month 12

  [Time Frame: 12 months] [Unit: ng/l or mg/l (CRP)] [Variable: Continuous]

  Assessed by routine clinical chemistry and ELISA

- Between-group changes in pancreatic fat between baseline and month 12

  [Time Frame: 12 months] [Unit: %] [Variable: Continuous]

  Assessed by magnetic resonance imaging (MRI)

- Between-group changes in flow-mediated dilation (FMD) between baseline and month 12

  [Time Frame: 12 months] [Unit: %] [Variable: Continuous]

Assessed by ultrasound in approximately half of the study population (n=75)

- Between-group changes in pulse-wave velocity (PWV) between baseline and month 12

  [Time Frame: 12 months] [Unit: m/s] [Variable: Continuous]

  Assessed by ultrasound in approximately half of the study population (n=75)

- Between-group values in FMD and PWV at month 12

  [Unit: % and m/s] [Variable: Continuous]

  Assessed by ultrasound in the whole population (n=150)

- Between-group changes in liver fat, HbA1c, and blood lipids in prespecified subgroups of type-2 diabetes status (yes/no), gender (male/female), individuals with NAFLD/without NAFLD at baseline, individuals with the CC vs CG/GG I148M genotype in PNPLA3, and in individuals with low respectively high dietary compliance based on dietary and lipogenic biomarkers changes (e.g. linoleic acid, DHA and palmitoleic acid changes from baseline to 12 months) between baseline and month 12

  [Time Frame: 12 months]

  We will assess whether response to diets in liver fat, HbA1c and blood lipids differ between gender, high vs low compliers, genotype, individuals with prediabetes or diabetes or those who have NAFLD/do not have NAFLD at baseline

4.3. Exploratory outcomes

- Between-group changes in plasma and imaging-derived fatty acids and fatty acid ratios in the lipogenic pathway (i.e.16:1n-7, 18:1n-9, 16:0, 14:0, 18:0 and 16:1n-7/16:0, 16:0/18:2n-6, 16:1n-7/18:2n-6) between baseline and month 12

  [Time Frame: 12 months] [Unit: %] [Variable: Continuous]

  Fatty acids are measured as percentage change of all fatty acids, thus using one unit for the above five fatty acids. Assessed by gas chromatography (GC) and proton spectroscopy, and use of bioinformatic modelling (untargeted) to identify responders and non-responders in liver fat reduction and improvement in glycemic control and blood lipids (i.e. personalized medicine approach)

- Between-group changes in plasma lipids (ceramides) using a targeted lipidomic approach between baseline and month 12

  [Time Frame: 12 months] [Unit: absolute and relative amounts] [Variable: Continuous]

  Lipids are measured using ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS)

4.4. Safety outcomes

Adverse events
Adverse events are reported at each clinic visit (after 6 and 12 months follow-up) using administered questionnaires with open-ended questions. Adverse events are categorized into 10 subcategories (ranging from gastrointestinal issues to serious adverse events) and will be presented as both the total number of events in each diet group (n/%) and separately for each of the 10 events (n/%) at both 6 months- and 12 months follow up.

Interim analysis
No interim analysis will be performed.

4.5. Co-interventions

Information on co-interventions such as medication use and dietary supplements (among others) are reported at each clinic visit (after 6 and 12 months follow-up) using administered questionnaires with open-ended questions. Alcohol intake will be assessed using the concentration of the phosphatidylethanol biomarker in fasting blood samples and physical activity (step count) will be assessed using pedometers.

5. Populations and subgroups to be analyzed

5.1 Populations

Intention to treat (ITT): All subjects randomized at baseline will constitute the ITT-population and will be the primary study population for the analyses. Missing data will be imputed as described in section 7 in this document.

Per-protocol (PP): All subjects completing the study period with complete data on the outcome of interest will constitute the PP-population. The PP-population will be the secondary study population for the analyses.

5.2 Subgroups

Further statistical analyses of the primary and secondary outcomes (liver fat, HbA1c and blood lipids) will be performed in the following prespecified subgroups:

Type-2 diabetes status: Subjects will be divided into two subgroups based on type-2 diabetes status (yes/no) of which the other subgroup ("no") will constitute of subjects without type-2-diabetes but with prediabetes as defined by a fasting plasma glucose between 5.6-6.9 mmol/L and/or an HbA1c value between 39-47 mmol/mol. Type-2 diabetes diagnosis is verified by a clinician using medical records.

Gender: Subjects will be divided into two subgroups based on gender (male/female).

NAFLD status: Subjects will be divided into two subgroups based on NAFLD status (yes/no) in which NAFLD is defined as a liver fat content exceeding 5.56 % of liver tissue.

PNPLA3 genotype: Subjects will be divided into two subgroups based on the I148M variant of the PNPLA3 gene (CC vs CG/GG genotype).

Compliers: Subjects will be divided into two subgroups based on dietary adherence (high vs low compliers), assessed using both dietary and lipogenic biomarker changes (e.g. linoleic acid, DHA and palmitoleic acid) between month 0 and month 12.

All subgroup analyses will be conducted as described for the analyses of the full study population.

6. Analyses

Normally distributed variables will be presented as mean ± SD and skewed distributed variables will be presented as median (IQR). Continuous variables will be tested for normality using the Shapiro-Wilk W test and skewed data ($W<0.95$) will be logarithmically transformed or analyzed non-parametrically, where appropriate. Homogeneity of variances between groups will be examined visually. Categorical variables will be presented as counts or percentages (%). An α-value of 0.05 is set as the significance level.

6.1. Primary outcome

For the primary outcome a General Linear Model (GLM) will be applied using Δliver fat (%) between month 12 and baseline as the dependent variable, treatment group as factor and type-2 diabetes status (yes/no), gender (male/female) and baseline liver fat content (%) as included covariates. The GLM will be applied for the ITT-population (defined above) as well as for the PP-population (defined above). Independent samples post-hoc t-tests will follow if between-group statistical significance is observed.

A sensitivity analysis will be conducted for the primary outcome in both the ITT- and PP-population, with weight change included as an additional covariate in the GLM.

Treatment effects will be presented as mean or median differences (%) with 95% confidence intervals and with corresponding p-values.

When assumptions of the GLM are not satisfied, the Willett´s residual method will be applied, followed by Mann-Whitney U post-hoc tests.

6.2 Secondary and exploratory outcomes

For secondary and exploratory outcomes GLMs will be applied using Δvalues between baseline and month 12 for each continuous variable (see point 4.2 and 4.3) as dependent variables and type-2 diabetes status (yes/no), gender (male/female) and baseline outcome values as included covariates in the model. Outcome values from 6 months follow-up will be presented descriptively.

The GLM will be applied for the ITT-population (defined above) as well as for the PP-population (defined above) for HbA1c, LDL cholesterol, triglycerides and systolic- and diastolic blood pressure. For the remaining secondary and exploratory outcomes, a PP-population will be used. Independent samples post-hoc t-tests will follow if between-group statistical significance is observed.

Treatment effects will be presented as mean or median differences (%) with 95% confidence intervals and with corresponding p-values.

When assumptions of the GLM are not satisfied, the Willett´s residual method will be applied (3), followed by Mann-Whitney U post-hoc tests.

6.3. Other statistical analyses that will be conducted

Pearson correlations (or Spearman rank correlation for non-normally distributed variables) between changes in serum fatty acids (presented as % of all fatty acids in that lipid compartment) and changes in liver fat content (%) and changes in HbA1c (mmol/mol) will be performed.

Pearson correlations (or Spearman rank correlation for non-normally distributed variables) between changes in liver fat content (%) and changes in HbA1c (mmol/mol), HOMA-IR, fasting plasma glucose (mmol/L), fasting serum insulin (mU/L), pancreatic fat (%), LDL-cholesterol (mmol/L), HDL-cholesterol (mmol/L), triglycerides (mmol/L), apoB (g/L) and apoA1 (g/L) will be performed.

7. Missing data

Missing numerical and categorical data (including both dependent- and explanatory variables) will be imputed using the technique of multiple imputation by chained equations (MICE). Categorical data will be coded into dummy variables. Number of missing values for each variable of which have been imputed will be presented as n/% for each group separately and in total.

8. References

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