Statistical Analysis Plan

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Index

1 Flowchart 3
2. Analysis Population/Study Population 4
2.1 Per-Protocol Population 4
2.2 Intention-To-Treat Population 4
3. Baseline Characteristics 5
4. Primary Endpoint – Risk Score 7
4.1 Definition of the Risk Score 7
5. Distribution and Normality Assessment 8
6. Delta Risk Score 8
7. Primary Analysis 9
8. Covariate Adjustment 10
9. Protocol Violations 10
10. Secondary endpoint – Tolerability and Compliance 10
11. Secondary endpoint – Circulating Biomarkers 11
12. Multiplicity 13
13. FADS1 Genotype – Exploratory Analysis ..... **Error! Bookmark not defined.**
14. Subgroup Analyses 13
15. Missing Data 13
16. Sensitivity Analyses 14
17. Safety Analyses 14
18. Diet and Physical Activity 14
18.1 Fish and Fish Oil 14
18.2 Physical Activity 14
19. Statistical Reporting 15
20. Statistical Software 15

1 Flowchart

- assessed for eligibility
- randomized (inclusion/exclusion criteria respected)
- allocated to treatment
- received treatment
- completed 12 weeks
- lost to follow-up
- major protocol deviations
- PP population
- ITT population

2. Analysis Population/Study Population

2.1 Per-Protocol Population

The Per-Protocol (PP) population will include all randomized participants who meet the eligibility criteria and have received the assigned intervention for at least 80% of the planned study duration, with no major protocol deviations considered to potentially affect the assessment of the primary endpoint.

2.2 Intention-To-Treat Population

The Intention-to-Treat (ITT) population will include all randomized participants, analyzed according to the treatment group to which they were originally randomized, regardless of treatment adherence or protocol deviations.

This analysis will be conducted as a sensitivity analysis of the primary endpoint.

3. Baseline Characteristics

Baseline characteristics will be summarized by treatment group and overall.

The three treatment groups will be defined as follows:

- A= 4 g/day fish oil
- B=10 g/day camelina oil
- C=control (non-enriched diet, only Vit. E)

Continuous variables will be reported using mean and standard deviation (SD), median and range, as appropriate, while categorical variables will be summarized using frequencies and percentages. Baseline characteristics showing a p-value <0.10 will be considered for inclusion as covariates in multivariable analyses, in accordance with the study protocol.

Table 1 – Baseline characteristics

Characteristics	A Fish oil (N = XXX)	B Camelina oil (N = XXX)	C Control (N = XXX)	Overall (N = XXX)	P-value[1]
Age – yr	XX [XX-XX]	XX [XX-XX]	XX [XX-XX]	XX [XX-XX]	
Sex – no. (%)	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)	
Diabetes mellitus – no. (%)	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)	
No	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)	
Yes, insulin-dependent	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)	
Yes, insulin-independent	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)	
Smoke – no. (%)					
No	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)	
Current	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)	
Ex smoker <=10 anni	XXX(XX)	XXX(XX)	XXX(XX)	XXX(XX)	
Ex smoker >10 anni	XXX(XX)	XXX(XX)	XXX(XX)	XXX(XX)	
Celiac disease – no. (%)	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)	
High Blood Pressure – no. (%)	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)	
Hypercholesterolemia – no. (%)	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)	
Obesity – no. (%)	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)	
Family History of Early-Onset Cerebrovascular Disease – no. (%)	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)	
Menopause – no. (%)	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)	
Other reason of the cardiovascular risk – no. (%)	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)	
Classification of Functional Capacity and Objective Assessment -no. (%) (attività fisica)					
Classe 1 = no limitations	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)	
Classe 2 = minor limitations	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)	
Classe 3 = significant limitation	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)	
Classe 4 = total incapacity	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)	
Systolic pressure – mm Hg	XXX ±XX	XXX ±XX	XXX ±XX	XXX ±XX	

Diastolic pressure – mm Hg	XX ±XX	XX ±XX	XX ±XX	XX ±XX
Heart rate – bpm	XXX ±XX	XXX ±XX	XXX ±XX	XXX ±XX
Wheight - kg	XX [XX-XX [XX-XXX] XXX]	XX [XX-XXX]	XX [XX-XXX]	
Height - cm	XX [XX-XX [XX-XXX] XXX]	XX [XX-XXX]	XX [XX-XXX]	
Waist Circumference - cm	XX [XX-XX [XX-XXX] XXX]	XX [XX-XXX]	XX [XX-XXX]	
ECG available				
Yes	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)
No	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)
Hematocrit - %	XX [XX-XX]	XX [XX-XX]	XX [XX-XX]	XX [XX-XX]
Hemoglobin – mg/dL	XX.X ±X.X	XX.X ±X.X	XX.X ±X.X	XX.X ±X.X
White blood cells – x 10^3/mm^3 or x 103/µl	XXX ±XXX	XXX ±XXX	XXX ±XXX	XXX ±XXX
Platelet counts – x 10^3/mm^3 or x 103/µl	XXX ±XXX	XXX ±XXX	XXX ±XXX	XXX ±XXX
Blood glucose level – mg/dl	XXX ±XXX	XXX ±XXX	XXX ±XXX	XXX ±XXX
HbAlc - % HB total	XX [XX-XX]	XX [XX-XX]	XX [XX-XX]	XX [XX-XX]
Total cholesterol – mg/dL	XXX ±XXX	XXX ±XXX	XXX ±XXX	XXX ±XXX
Cholesterol LDL – mg/dL	XX ±XX	XX ±XX	XX ±XX	XX ±XX
Cholesterol HDL – mg/dL	XX ±XX	XX ±XX	XX ±XX	XX ±XX
Hypertriglyceridemia – mg/dL	X.XX ±X.XX	X.XX ±X.XX	X.XX ±X.XX	X.XX ±X.XX
Serum Creatinine – mg/dL	XX [XX-XX]	XX [XX-XX]	XX [XX-XX]	XX [XX-XX]
eGRF – ml/min/1.73 m2	XX ±XX	XX ±XX	XX ±XX	XX ±XX
Who does the patient live with – no. (%)				
Alone	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)
Spouse	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)
Family	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)
Other	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)
Education – no. (%)				
Elementary school	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)
Middle school	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)
High school	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)
Accademy	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)
Bachelor's degree	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)
Master degree	XXX(XX)	XXX(XX)	XXX(XX)	XXX(XX)
PhD	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)
Activity – no. (%)				
Retiree	XXX(XX)	XXX(XX)	XXX(XX)	XXX(XX)
Stay-at-home parent	XXX(XX)	XXX(XX)	XXX(XX)	XXX(XX)
Employee	XXX(XX)	XXX(XX)	XXX(XX)	XXX(XX)
Self-employed professional	XXX (XX)	XXX (XX)	XXX (XX)	XXX (XX)

[1]Chisq-test for categorical variables and Kruskal-Wallis test for continuous variables, as appropriate.

4. Primary Endpoint – Risk Score

4.1 Definition of the Risk Score

The primary endpoint will be the lipidomic/inflammation-derived risk score measured at baseline and at 12 weeks. The construction and calculation of the risk score will be defined prior to database lock and treatment unblinding.

If the study-specific risk score is not available according to the predefined timeline, the validated CERT2 risk score will be used as specified in the study protocol. CERT2 score ranges from 0 to 12 points: 0-3 points low risk; 4-6 points moderate risk; 7-8 points increased risk; 9-12 points high risk.

Table 2 – CERT2 scoring system				
	points			
CERT2 score component	Q1	Q2	Q3	Q4
Cer(d18:1/24:1)/Cer(d18:1/24:0)	0	1	2	3
Cer(d18:1/16:0)/PC(16:0/22:5)	0	1	2	3
Cer(d18:1/18:0)/PC(14:0/22:6)	0	1	2	3
PC(16:0/16:0)	0	1	2	3

CERT2 score per treatmentgroup at baseline and 12 weeks FU will be assessed.

Table 3						
	$RiskScore_{baseline}$			$RiskScore_{12weeks}$		
	A Fish oil (N = XXX)	B Camelina oil (N = XXX)	C Control (N = XXX)	A Fish oil (N = XXX)	B Camelina oil (N = XXX)	C Control (N = XXX)
N (%)						
Min – Max						
Mean (SD)						
Median [Q1,Q3]						

5. Distribution and Normality Assessment

The normal distribution will be verified using appropriate graphical representations (for example histograms and/or Q-Q plot) and, where appropriate, the Shapiro Wilk test.

The choice between parametric and non-parametric statistical methods will be based on the distribution of the outcome and the assumptions of the corresponding statistical model.

ANOVA will be used for a parametric comparison and/or the Kruskal-Wallis test for a nonparametric comparison.

6. Delta Risk Score

The change in risk score from baseline to 12 weeks will be calculated as:

$$\Delta_{RiskScore} = \boldsymbol{RiskScore}_{12weeks} - \boldsymbol{RiskScore}_{baseline}$$

Negative values therefore indicate a decrease in the risk score from baseline to 12 weeks, whereas positive values indicate an increase.

The change in risk score will be summarized by treatment group using mean and SD and/or median and interquartile range (IQR), as appropriate.

The distribution of the change in risk score will also be assessed using graphical methods and, where appropriate, the Shapiro-Wilk test.

As a supportive analysis, the change in risk score will be compared across the three treatment groups. One-way analysis of variance (ANOVA) will be used when the assumptions of the model are adequately met; otherwise, the Kruskal-Wallis test will be considered.

The estimated differences between treatment groups will be reported with corresponding 95% confidence intervals and two-sided p-values, where applicable.

7. Primary Analysis

The primary analysis will compare the effect on risk score in the 3 groups of treatment groups on the risk score at 12 weeks using analysis of covariance (ANCOVA).

In the primary ANCOVA model, the 12-week risk score will be the dependent variable and treatment group will be included as the main fixed effect. The baseline risk score will be included as a covariate. Additional baseline covariates may be included according to the prespecified criteria described in Section 8.

The primary model can be expressed as:

$$\boldsymbol{RiskScore_{12weeks} = Treatment + RiskScore_{baseline} + Covariates}$$

$$\boldsymbol{\Delta RiskScore = Treatment + Covariates}$$

Table 4 - Risk score at baseline and 12 weeks by treatment group

	A Fish oil (N = XXX)	**B** Camelina oil (N = XXX)	**C** Control (N = XXX)
$\boldsymbol{RiskScore_{baseline}}$	XX [XX-XX]	XX [XX-XX]	XX [XX-XX]
$\boldsymbol{RiskScore_{12weeks}}$	XX [XX-XX]	XX [XX-XX]	XX [XX-XX]
Δ_RiskScore	XX [XX-XX]	XX [XX-XX]	XX [XX-XX]

Treatment effects will be estimated as adjusted mean differences between treatment groups, together with corresponding 95% confidence intervals and two-sided p-values.

Three pairwise treatment comparisons will be reported:

1. Fish oil (A) vs Control (C)
2. Camelina oil (B) vs Control (C)
3. Fish oil (A) vs Camelina oil (B)

The comparison between treatment A and treatment B will assess whether the two active interventions differ in their effect on the primary endpoint. Statistical significance will be assessed using a two-sided alpha level of 0.05.

Table 5 - Adjusted treatment effects on the primary endpoint

	Adjusted mean difference	95% CI	P-value
Fish oil (A) vs Control (C)			
Camelina (B) oil vs Control (C)			
Fish oil (A) vs Camelina Oil (B)			

8. Covariate Adjustment

Covariates considered for multivariable adjustment will include the baseline value of the outcome and baseline characteristics showing a p-value <0.10 across treatment groups, in accordance with the protocol. The final set of covariates will be defined prior to database lock and treatment unblinding.

9. Protocol Violations

The primary analysis will be conducted on the pre-protocol population, that is, on the randomized subjects who received the assigned treatment for at least 80% of the planned supplementation period and without major protocol violations.

Table 6. Major Protocol violations				
	A Fish oil (N = XXX)	B Camelina oil (N = XXX)	C Control (N = XXX)	Overall (N=XXX)
Treatment adherence <80% – no. (%)	XX/XXX (X.XX%)	XX/XXX (X.XX%)	XX/XXX (X.XX%)	XX/XXX (X.XX%)
Eligibility deviation	XX/XXX (X.XX%)	XX/XXX (X.XX%)	XX/XXX (X.XX%)	XX/XXX (X.XX%)
Follow-up deviation	XX/XXX (X.XX%)	XX/XXX (X.XX%)	XX/XXX (X.XX%)	XX/XXX (X.XX%)
*Other deviation**	XX/XXX (X.XX%)	XX/XXX (X.XX%)	XX/XXX (X.XX%)	XX/XXX (X.XX%)

*in case of other deviations.

10. Secondary endpoint – Tolerability and Compliance

Test the tolerability for use of the 2 types of oils. Categorical tolerability and adherence outcomes will be summarized as frequencies and percentages and compared across the three treatment groups using the chi-square test or Fisher's exact test, as appropriate.

Table 7. Treatment adherence and tolerability				
	A Fish oil (N = XXX)	B Camelina oil (N = XXX)	C Control (N = XXX)	P Chi-sq
Interruption of treatment- no. (%)	XX/XXX (X.XX%)	XX/XXX (X.XX%)	XX/XXX (X.XX%)	
Reason for interruption				
Adverse event	XX/XXX (X.XX%)	XX/XXX (X.XX%)	XX/XXX (X.XX%)	
Flavour event	XX/XXX (X.XX%)	XX/XXX (X.XX%)	XX/XXX (X.XX%)	
Other	XX/XXX (X.XX%)	XX/XXX (X.XX%)	XX/XXX (X.XX%)	

11. Secondary endpoint – Circulating Biomarkers

The protocol specifies any improvement in lipid, inflammatory, and glycemic profiles as a secondary endpoint.

- Lipid profile
  - Total cholesterol
  - LDL
  - HDL
  - Triglycerides

- Inflammatory biomarkers

  White blood cell count

- Glycemic profile
  - Glicemia
  - HbAlc

- Other
  - Ematocrit
  - Hb
  - Piastrine
  - Creatinine
  - egfr

Table 8. Circulating biomarkers at baseline and 12 weeks

Biomarker	A baseline	A 12w	B baseline	B 12w	C baseline	C 12w
Total cholesterol						
LDL						
HDL						
Triglycerides						
***						
Glucose						
HbA1c						

For each biomarker:

$$Biomarker_{12weeks} = Treatment + Biomarker_{baseline} + Covariates$$

Changes in circulating lipid, inflammatory and glycemic biomarkers from baseline to 12 weeks will be evaluated by treatment group.

Continuous outcomes will primarily be analyzed using ANCOVA, with the 12-week biomarker value as the dependent variable and treatment group and baseline biomarker value as explanatory variables.

Adjusted mean differences between treatment groups will be reported with 95% confidence intervals and two-sided p-values.

Table 9. Adjusted treatment effects on secondary biomarkers

Biomarker	Comparison Treatment	Adjusted mean difference	95% CI	P-value
Total cholesterol				
LDL				
HDL				
Triglycerides				
***				
Glucose				
HbA1c				

12. Multiplicity

No formal adjustment for multiplicity will be applied to secondary and exploratory endpoints. P-values for secondary and exploratory analyses will therefore be interpreted descriptively and in the context of the corresponding effect estimates and 95% confidence intervals.

13. Subgroup Analyses

Prespecified subgroup analyses will be performed according to age (<65 vs ≥65 years), sex and other to be defined.For each subgroup, treatment effects will be estimated separately and the statistical evidence for effect modification will be assessed by including the corresponding treatment-by-subgroup interaction term in the model.

Table 10A. Prespecified subgroup analyses of the primary endpoint - Age

Subgroup	Comparison	Adjusted mean difference	95% CI	Interaction P-value global
Age <65	A vs C			
Age ≥65	A vs C			
Age <65	B vs C			
Age ≥65	B vs C			
Age <65	A vs B			
Age ≥65	A vs B			

Table 10B. Prespecified subgroup analyses of the primary endpoint - sex

Subgroup	Comparison	Adjusted mean difference	95% CI	Interaction P-value global
Sex: Male	A vs C			
Sex: Female	A vs C			
Sex: Male	B vs C			
Sex: Female	B vs C			
Sex: Male	A vs B			
Sex: Female	A vs B			

14. Missing Data

No missing imputation will be performed.

15. Sensitivity Analyses

A sensitivity analysis will be performed in the Intention-to-Treat population, including all randomized participants analyzed according to their randomized treatment allocation. The same primary endpoint model used for the Per-Protocol analysis will be applied.

16. Safety Analyses

The protocol calls for the collection of adverse events, serious adverse events, and specific categories of events.

Table 11. Adverse events by treatment group			
Safety outcome	A Fish oil (N = XXX)	B Camelina oil (N = XXX)	C Control (N = XXX)
Any AE			
Serious AE			

17. Diet and Physical Activity

Changes in dietary intake and physical activity collected by means of self reported questionnaires will be summarized descriptively and explored according to treatment group.

17.1 Fish and Fish Oil

The same analysis conducted in paragraph 14 for subgroups, but limited solely to the group of patients who took fish oil (A), comparing those who never eat fish, those who eat it 1–2 times a week, and those who eat it more than 3 times a week

Table 12° - fish consumption and fish oil effect			
Subgroup	Adjusted mean difference of biomarker score	95% CI	Interaction P-value global
Fish (mai)			
Fish (1-2 volte)			
Fish (>=3 volte)			

17.2 Physical Activity

By means of the SQUASH questionnaire the physical activity of participants will be assessed by means of “metabolically equivalent tasks” MET-hours per week and any interaction with the CERT2 levels will be compared.

Table 12 b – Effect of physical activity on biomarkerscore			
Subgroup	Adjusted mean difference of biomarker score	95% CI	Interaction P-value global
Lowest tertile of physical activity			
Middle tertile of physical activity			
Highest tertile of physical activity			

18. Statistical Reporting

All statistical tests will be two-sided. Statistical significance for the primary endpoint will be assessed at the 5% significance level. Estimates will be reported with corresponding 95% confidence intervals. Continuous variables will be summarized using appropriate measures of central tendency and dispersion, while categorical variables will be presented as counts and percentages.

No interim efficacy analysis is planned.

19. Statistical Software

All statistical analyses will be performed using R statistical software (R Foundation for Statistical Computing, Vienna, Austria), version to be specified at the time of analysis. The statistical packages used for the analyses will be documented in the final statistical report.