Statistical Analysis Plan for iPROACT-pilot

Aim

The aim of iPROACT-pilot is to explore the short-term effects of daily oral supplementation with indole-3-propionic acid (IPA) in healthy adults. Results will provide an overview of potential beneficial effects and side effects within a range of three different doses of supplementation. Such insights can guide the design of future trials with longer supplementation duration and of higher statistical power in relevant populations.

Hypotheses

We hypothesize that oral supplementation with IPA will:

- reduce inflammation
- increase endogenous production of neurotrophic factors
- improve metabolic syndrome-related risk factors
- minimize oxidative stress and prevent oxidative damage

We expect the effects to be dose-dependent with no side effects even in the highest dose used. Dose-dependency may be related to the dose expressed mg IPA per kg body weight per day.

We expect the potential of IPA to demonstrate an effect on a given outcome to be highest among individuals with abnormal baseline values of that given outcome.

Lastly, we hypothesize that the effect sizes may be largest among individuals with lower baseline levels of IPA.

Design

iPROACT-pilot is a randomized, double-blind, placebo-controlled trial with four parallel arms (daily IPA dose: placebo vs. 50 mg vs. 120 mg vs. 500 mg) investigating the effects of two weeks of oral supplementation with IPA among a minimum of 64 healthy adults (see “Eligibility Criteria” of ClinicalTrials.gov ID NCT06674018 for inclusion and exclusion criteria). Additional healthy adults wishing to participate before the last inclusion date of the study, are allowed to do so. Study endpoints are measured at baseline (day 1: first day of supplementation) and then again at day 15 (see section “What is the study measuring?” of ClinicalTrials.gov ID NCT06674018 for timing of fecal samples and maize test). Block-randomization is used with random block sizes of 4 or 8 and an allocation ratio of 1:1:1:1. No stratification criteria are used. Participants as well as all study personnel are blinded towards the intervention. Furthermore, due to the use of unique IDs on each capsule bottle, it is impossible for participants or study personnel to guess which participants belong to the same arm (see “Masking Description” of ClinicalTrials.gov ID NCT06674018 for more details). Group formation as well as the identity of each group will only be revealed to the researchers performing statistical analyses after the respective data have been cleaned and quality checked.

Populations

As the main objective of this study is to explore the biological effects of IPA supplementation, the primary analyses will be based on a per protocol population.

The per protocol population includes all study participants that completed the study with satisfactory compliance. Satisfactory compliance is defined as intake of more than 85% of the intended capsules (two per day for each study day before the follow-up visit) with IPA or placebo. Containers with remaining capsules are returned at the follow-up visit. For each outcome, only complete cases are used in the analyses, meaning that information on follow-up values, baseline values and any applied covariates need to be present for the participant to be included in evaluation of the given outcome. As practical or technical challenges may affect collection of information on some but not other parameters, slight variations may arise in the populations used to evaluate the effect of IPA on different outcomes.

Should suspicion of potential biases arise, an intention-to-treat analysis will also be performed.

Missing data

The primary analyses are based on a per-protocol population of complete cases, meaning that participants with missing data for a given outcome will be excluded from its analysis.

Nevertheless, results from laboratory analyses that fall below the limit of quantification will be imputed using a reasonable value below that limit. For example, our laboratory has a limit of quantification of 0.4 mg/L for C-reactive protein (CRP). Results reported as <0.4 mg/L will be replaced by 0.3 mg/L.

In case of an intention-to-treat analysis or other exploratory analyses, missing data may be imputed using multiple imputation by chained equations (MICE). Imputations will be based on predictive mean matching for numeric variables and on logistic regression for two-level factors.

Outcomes

The primary outcome is defined as the population of $FoxP3^{+}CD25^{+}CD127^{-}$ regulatory T cells (Tregs) expressed as a percentage of single, live $CD3^{+}CD4^{+}CD8^{-}$ lymphocytes analysed in freshly isolated peripheral blood mononuclear cells (PBMCs) using a Symphony A3 flowcytometer. Plasma concentration of brain-derived neurotrophic factor (BDNF) is defined as a second primary outcome.

iPROACT-pilot was initiated to allow for selection of the optimal IPA dose for two future randomized controlled trials (RCTs): one with patients with optic neuritis and one with patients with multiple sclerosis. Therefore, the primary outcomes are defined based on their relevance for neuroinflammation. A negative result in one of the primary outcomes does not invalidate potential findings in other outcomes. A full list of predefined outcomes is presented in the section “What is the study measuring?” of ClinicalTrials.gov ID NCT06674018. Funding has not been acquired for analyses of all predefined outcomes. This statistical analysis plan focuses on 12 prioritized primary and secondary efficacy

outcomes grouped according to the hypothesis they address. Additional efficacy and safety outcomes will be analysed in more exploratory analyses studying either the effects of IPA supplementation or exploring the associations between the measured outcomes (ex. association between diet and immunity).

Inflammation

Primary

- Tregs in PBMCs defined as the $FoxP3^+CD25^+CD127^-$ population and expressed as a percentage of single, live $CD3^+CD4^+CD8^-$ lymphocytes (theoretical range: 0-100)

Secondary

- Th1/Th2 ratio in PBMCs, where Th1 cells are defined as the $CXCR3^+CCR4^-CCR6^-CCR10^-$ non-Treg population and expressed as a percentage of single, live $CD3^+CD4^+CD8^-CD45RA^-$ lymphocytes and Th2 cells are defined as the $CXCR3^-CCR4^+CCR6^-CCR10^-$ non-Treg population and expressed as a percentage of single, live $CD3^+CD4^+CD8^-CD45RA^-$ lymphocytes
- Th17/mTreg ratio in PBMCs, where Th17 cells are defined as the $CXCR3^-CCR4^+CCR6^+CCR10^-$ non-Treg population and expressed as a percentage of single, live $CD3^+CD4^+CD8^-CD45RA^-$ lymphocytes and mTreg (memory Tregs) are defined as the $FoxP3^+CD25^+CD127^-CD45RA^-$ population and expressed as a percentage of single, live $CD3^+CD4^+CD8^-CD45RA^-$ lymphocytes
- Th17.1 cells in PBMCs defined as the $CXCR3^+CCR4^-CCR6^+CCR10^-$ non-Treg population and expressed as a percentage of single, live $CD3^+CD4^+CD8^-CD45RA^-$ lymphocytes
- CRP (mg/L)

We hypothesize that supplementation with IPA will result in increased abundance of $FoxP3^+$Tregs as well as reduced Th1/Th2 ratio, reduced Th17/mTreg ratio, reduced abundance of Th17.1 cells and reduced CRP.

Neurotrophic factors

- Plasma concentration of BDNF (second primary outcome)

We hypothesize that supplementation with IPA will increase plasma BDNF.

Metabolic syndrome-related risk factors

Secondary

- Fasting plasma triglycerides (mmol/L)
- Fasting plasma non-HDL cholesterol (mmol/L)
- Fasting plasma C-peptide (pmol/L)
- Fasting plasma glucose (mmol/L)

We hypothesize that supplementation with IPA will lead to reduced levels of triglycerides, reduced levels of non-HDL cholesterol, reduced fasting C-peptide and reduced fasting glucose. As the study is performed in healthy individuals, we expect a drop in C-peptide to reflect improved insulin sensitivity and not reduced beta-cell function.

Oxidative stress
Secondary

- Urinary 8-iso-prostaglandin F2α (maker of lipid oxidation)
- 8-hydroxydeoxyguanosine (marker of DNA oxidation measured in urine or buffy coats)

We hypothesize that supplementation with IPA will lead to reduced levels of 8-iso-prostaglandin F2α and 8-hydroxydeoxyguanosine.

Statistical analysis

Descriptive statistics
Descriptive statistics of numerical variables will be presented as mean (±SD) for normally distributed variables and median [1st quartile, 3rd quartile] for non-normally distributed variables. Normality will be assessed using the Shapiro-Wilk normality test. Categorical variables and ordinal variables with few categories will be presented as number (%). Ordinal variables with many categories will either be presented as median [1st quartile, 3rd quartile] or categories with few observations will be merged to provide meaningful groupings allowing for presentation as number (%).

Analysis methods
Results will be analysed according to a superiority framework employing a significance level of 5%. Analysis of covariance (ANCOVA) will be used to quantify the effects of IPA on the above prioritized efficacy outcomes (primary efficacy analyses). Log-transformed outcome values at follow-up are defined as the dependent variable. For each biomarker, independent variables include treatment arm (the placebo group will be set as reference) and log-transformed baseline values of the investigated outcome. The primary analysis is defined as the comparison between the treatment arm with the highest IPA dose and placebo in the model based on data from the entire per protocol population (all four groups).

Furthermore, an Emax model will be used to investigate the dose-response relationship between the administered dose of IPA, expressed in mg per kg of body weight, and its biological effects.

In secondary analyses, a relationship between treatment effects and baseline serum concentrations of IPA as well as an interaction between treatment arm and baseline values of the investigated outcomes (relevant cut-offs will be defined) will be explored.

Signatures

Author of statistical analysis plan

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