

Statistical Analysis Plan for iPROACT-pilot part 2

Aim

The aim of iPROACT-pilot part 2 is to explore the short-term effects of oral supplementation with indole-3-propionic acid (IPA) taken twice daily (500 mg in the morning and 500 mg in the evening) in healthy adults. Results will provide an overview of potential beneficial effects and side effects that 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 potential of IPA to demonstrate an effect on a given outcome to be highest among individuals with abnormal baseline values of that given outcome.

Design

iPROACT-pilot is a randomized, double-blind, placebo-controlled trial with two parallel arms investigating the effects of two weeks of oral supplementation with 500 mg of IPA twice daily (total daily dose: 1000 mg IPA) among a minimum of 32 healthy adults. Additional eligible healthy adults wishing to participate before the last inclusion date of the study, are allowed to do so. The study will include healthy women and men, ≥ 18 and ≤ 65 years of age that are deemed mentally and physically able to participate. Exclusion criteria are:

- Diagnosis of gut-, heart-, liver-, kidney or immune-related disorders
- Use of antibiotics within the last month
- Pregnancy, lactation or childbirth within the last five months
- Use of prescription medication

Block-randomization is used with random block sizes of 2 or 4 and an allocation ratio of 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 container, it is impossible for participants or study personnel to guess which participants belong to the same arm. 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.

Most study endpoints are measured on day 1 prior to initiation of supplementation (day 1: first day of supplementation) and then again on day 15. Blood samples are collected in a fasting state between 8-10 AM and again 1.5 hours after intake of an IPA/ placebo dose on day 1 and day 15. Fecal samples are collected before initiation of supplementation (earliest 48 hours prior to day 1 visit), shortly after initiation of supplementation (day 3 or soonest thereafter) and towards the end of supplementation (earliest 48 hours prior to day 15 visit). A maize test is used to estimate transit time starting five days prior to day 1 and then repeated 5 days prior to day 15.

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 (four per day for each study day before the follow-up visit at day 15) 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. The concentration of brain-derived neurotrophic factor (BDNF) in platelet-free plasma is defined as a second primary outcome.

iPROACT-pilot was initiated to gain necessary insight prior to performance of two future randomized controlled trials (RCTs): one with patients with optic neuritis and one with patients with multiple sclerosis. Therefore, the primary outcomes of iPROACT-pilot and iPROACT-pilot part 2 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 at ClinicalTrials.gov. 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

- Concentration of BDNF in platelet-free plasma (second primary outcome)

We hypothesize that supplementation with IPA will increase BDNF in platelet-free plasma.

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 F2 α (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 F2 α and 8-hydroxydeoxyguanosine.

Statistical analysis

Descriptive statistics

Descriptive statistics of numerical variables will be presented as mean (\pm 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 and log-transformed baseline values of the investigated outcome.

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.

Approvals

This statistical analysis plan was authored by PhD, MSc Moschoula Passali and approved by principal investigator & sponsor Prof, DMSc Jette Frederiksen the 16th of July 2025.