STATISTICAL ANALYSIS PLAN

The effect of diet on immune and vaccine responses in people living with obesity in transitioning communities.

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BACKGROUND

The prevalence of overweight and obesity is rapidly increasing globally. Obesity is associated with immune dysfunction leading to an increased risk of severe infectious illnesses and diminished vaccine effectiveness. Our previous studies in the Kilimanjaro region have shown that diet has a pronounced effect on the function of the immune system in healthy individuals. The traditional high plant-based fiber and polyphenol diet and a locally consumed fermented banana brew were anti-inflammatory. However, it is still unclear to what extent dietary variation directly or indirectly influences the host immune defense in individuals who live with obesity and whether certain dietary interventions may enhance immune responses and improve vaccine efficacy.
The current study ('TransInf') aims to fill these gaps by establishing the cause-and-effect relationship between specific nutritional factors and immune responses to common viral and bacterial infections, as well as the response to vaccines, in people living with obesity in a transitioning societal environment in Tanzania.

STUDY DESIGN AND ANALYSIS POPULATION

The study combines a cross-sectional (dataset 1) design with a randomized (dataset 2), open-label, proof-of-concept nutritional intervention study. Tanzanians (n=100) aged 30 to 60 years with a BMI >25 Kg/M^2(Obese) at screening will be recruited, together with age and sex-matched Tanzanians (n=50) with a BMI 18.5–24.9 Kg/M^2 (normal weight controls). Ninety of the overweight/obese participants will be randomly allocated to one of the following 3 arms: i) high plant-based fiber and polyphenol diet (n=30); ii) daily intake of fermented banana beverage (n=30); iii) continuation of usual diet (obese controls; n=30). The dietary intervention will take place for six weeks. At visit 2 (baseline), blood and stool samples will be collected for functional immune assays (ex vivo whole blood stimulation) and a set of omics technologies (baseline sampling, 100 overweight participants, and 50 lean participants). At week 4 (visit 3), blood and stool samples will be collected from 90 participants in the intervention arms (interventional sampling). To assess the effects of the dietary intervention on vaccine responses, participants will receive a conjugated pneumococcal vaccine (Prevenar13, Pfizer) and tetanus toxoid vaccine (Biological E Limited, India) at week 4 and continue with their diets for another 2 weeks. The effect of diet on vaccine response will be assessed by measuring antibody titers 4 weeks after vaccine administration (visit 4). The following measurements will be performed on the collected biomaterials: metagenomics (changes in the gut microbiome), plasma proteomics (changes in the inflammatory and cardio-metabolic proteins expression), *ex vivo* whole blood stimulation (to assess the capacity of the circulating immune cells to produce cytokines to different microbial ligands), immunoglobulin binding to the gut microbiome, vaccines responses, immune cell telomere length, and whole blood transcriptome.
The study protocol was submitted for approval to the Kilimanjaro Christian Medical College Research Ethics and Review Committee (CRERC) as well as to the National Institute for Medical Research (NIMR), in Dar es Salaam, Tanzania. The study has been registered at the ISRCTN trial registry.

PATIENT BASELINE DEMOGRAPHICS AND CROSS SECTIONAL COMPARISON

Baseline demographics will be presented as percentages for categorical variables and as mean ± standard deviation (SD) or median with interquartile range (IQR) for continuous variables, depending on the normality of their distribution.

For dataset1, quantitative variables following a normal distribution will be compared between groups (obese vs lean) using the Student's t-test, while non-normally distributed variables will be compared using the Mann-Whitney U test. Categorical variables will be compared using Fisher's exact test or chi-square test.

Linear models will be utilized for differential expression analysis of plasma proteins and metabolites between groups.

For correlation analysis, Pearson partial correlation coefficients will be obtained to control for confounding variables.

Transcriptome data will be analyzed using DESeq2 for differential gene expression, with enrichment gene set analysis performed using Fisher's exact test or hypergeometric test.

For microbiome analysis, bioinformatics tools will be applied to characterize gut microbiota profiles, with LEfSe analysis for enriched taxa and metagenomics markers. Alpha and beta diversity will be estimated, and non-parametric statistical tests (Kruskal-Wallis and pairwise Wilcoxon rank-sum tests) will be used to compare relative abundances of microbial/fungal taxa between groups, with FDR correction for multiple testing.

Additional explorative analysis will be done using state-of-the-art analysis tools.

ANALYSIS FOR THE STUDY ENDPOINTS

Primary trial endpoint

According to the study protocol, the primary effect endpoints are the:

1. Changes from baseline in innate and adaptive cytokine responses to microbial ligands at week 4 within and between the intervention arms.
2. Differences in anti-pneumococcal and anti-tetanus antibody responses between the intervention arms at week 8.

Analyses of the primary outcome will be done as follows:

First Primary endpoint (cytokine responses).

i) To assess **within-group changes** in cytokine responses within the same intervention arm at baseline (week 0), during intervention (week 4), and post-vaccination (week 8).
   - If the data will be normally distributed, we will employ a paired T-test to compare differences in cytokine responses across the three time points
   - If the data is not normally distributed, when suitable, we will perform transformation to achive normality and follow the analysis plan for normally distributed data. If not possible, we will then employ a non-parametric Wilcoxon signed-rank test to compare differences in cytokine responses across the three time points.

The analysis of the primary endpoint will be further confirmed by linear mixed models including fixed effect as time point, random effect as individual and additional metadata co-variates.

ii) To assess **between-group differences**, Kruskal Wallis test (for not-normally distributed data) or one-way ANOVA (for normally distributed data) with post hoc tests if significant will be achieved to compare cytokine responses across the three arms.

Second primary endpoint (vaccine responses).

Pneumococcal responses. We will measure the serotype-specific pneumococcal antibody concentrations of the 13 pneumococcal serotypes included in the PCV13 vaccine. The following three outcome measures will be analysed:

- Absolute concentrations of serotype-specific pneumococcal antibodies at week 8 will be compared between arms using ANOVA (for normally distributed data) or Kruskal-Wallis test (for non-normally distributed data) with post hoc tests if significance will be achieved. If necessary, data will be transformed (e.g. logarithmic transformation).
- Fold increase in serotype-specific pneumococcal antibody concentrations between week 4 and week 8 will be compared between arms using ANOVA or Kruskal-Wallis test with post hoc tests if significant will be achieved.
- The proportion of serotypes with a positive antibody response at week 8 will be analyzed. A positive response is defined as an IgG level of ≥1.0 µg/mL or a ≥4-fold increase from baseline. In addition, a less stringent IgG level of ≥0.35 µg/mL or a ≥2-fold increase baseline will be used.

For all comparisons, a two-sided p-value < 0.05, after correction for multiple tests using FDR/Bonferroni will be considered statistically significant for all tests.

Secondary study endpoints

According to the study protocol, the secondary effect endpoints are the:

1. Changes from baseline in inflammatory and cardiometabolic plasma proteome at week 4 across the 3 study arms.
2. Changes from baseline in whole blood transcriptome and immune gene regulation by long non-coding RNAs at week 4 across the 3 study arms.
3. Changes from baseline in untargeted plasma metabolome across the 3 arms.
4. Change from baseline in tetanus and pneumococcal specific T-cell responses.
5. Changes from baseline in the composition of the gut microbial, sIgA-binding of gut commensals ex vivo and systemic antibody levels to gut commensals.
6. Changes from baseline in telomere length at week 4 across the 3 arms.
7. Changes from baseline in DNA methylation of peripheral blood mononuclear cells (PBMCs) at 4 weeks across the 3 arms.
8. Differences in vaccine-induced changes in whole blood transcriptome between the 3 arms.

Analyses of the secondary outcome measures will be done as follows:

- Dimensionallity reduction (such as Principal component analysis) will be performed to explore omics data
- Wilcoxon signed-rank tests or paired T-tests for individual parameters
- Linear mixed models including fixed effect as time point, random effect as individual and additional metadata co-variates

- Transcriptome data will be analyzed using DESeq2 for differential gene expression, with enrichment gene set analysis performed using Fisher's exact test or hypergeometric test.
- For microbiome analysis, bioinformatics tools will be applied to characterize gut microbiota profiles, with LEfSe analysis for enriched taxa and metagenomics markers. Alpha and beta diversity will be estimated, and non-parametric statistical tests (Kruskal-Wallis and pairwise Wilcoxon rank-sum tests) will be used to compare relative abundances of microbial/fungal taxa between groups, with FDR correction for multiple testing.
- For telomer and tetanus specific T cells assessments, paired t-tests comparing measured variables (telomer length or %IFN-g+ specific T cells) between baseline and week 4 will be performed. Comparison between the 3 arms will be assessed with Kruskal-Wallis tests.