Statistical analysis plan (SAP)

Section 1: Administrative information

1.1	Title and trial registration number	Proteomic Analysis on Myopic Children using Orthokeratology Lenses with Various Effects of Myopic Control: The ProMOC study ClinicalTrials.gov Identifier: NCT06647472 The title have been clarified further to enhance transparency.
1.2	Names, affiliations and roles of SAP contributers	Barha Aftab, Pregraduate medical student Department of Opthalmology, Vejle Hospital Lasse Cehofski, MD, PhD, Associate Professor of Ophthalmology Department of Ophthalmology, Aalborg Hospital Flemming Møller, MD, Consultant, PhD, DMsc, Associate Professor Department of Opthalmology, Vejle Hospital Trine M. Jakobsen, MD, PhD, Postdoc Department of Opthalmology, Vejle Hospital Lou-Ann C. Andersen, MD, PhD student Department of Opthalmology, Vejle Hospital
1.3	Principal investigator/project lead	Principal investigator MD, PhD. student Lou-Ann Christensen Andersen or her delegate stud.med. Barha Aftab
1.4	Statistician/data analyst	Lasse Cehofski, MD, PhD, Associate Professor of Ophthalmology Department of Ophthalmology, Aalborg Hospital Bent Honoré, MD, PhD, Associate Professor of Biomedicine Department of Biomedicine, Aarhus University
1.5	Reference to protocol version being used	17-08-2024, Version 2 Aproved by the Regional Committees on Health Research Ethics for Southern Denmark
1.6	SAP version and revision history	Version 1, 21-03-2025
1.7	Date for approval of final SAP version	21-03-2025

1.8	Timeframe for conducting the proposed analysis	01-09-2025

Section 2: Introduction

2.1	Describe briefly background, research questions and rationale behind the study	Orthokeratology lenses (also known as OK lenses or ortho-k lenses) have proven to be effective in reducing myopia progression by slowing axial length elongation, though the efficacy varies among children. The key objective of the study is to understand the biochemical processes that make ortho-k lenses more effective for some individuals than for others. With this knowledge, we hope to identify possible molecules present on the ocular surface for those who are currently experiencing less favorable treatment efficacies.
2.2	Describe briefly objectives and/or hypotheses	<u>Purpose</u> The purpose of this exploratory study is to assess ocular biochemical alterations by analyzing the composition of proteins deposited on ortho-k lenses in children undergoing ortho-k treatment. <u>Endpoint</u> The primary aim is to investigate differences in protein levels in children using ortho-k lenses with varying effects on myopia control. <u>Hypothesis</u> There is a significant difference in the expression of molecules in children with various effects of myopia control.

Section 3: Study methods

3.1	<u>Study design</u> Describe type of study (i.e. experimental/observational, parallel group/cross over, singlecenter/multicenter ect.) and describe briefly interventions	Exploratory cross-sectional study.
3.2	<u>Randomization details</u> (if applicable) Describe randomization i.e. allocation ratio, potential factors randomization will be stratified for and describe how and when randomization will be performed	No randomization.
3.3	<u>Sample size</u> Describe calculation of sample size or reference to	We aim to include a total of 25 children. As this is the first study of its kind, no prior data are available to facilitate a power calculation.

	sample size calculation in study protocol	
3.4	Hypotheses framework Describe hypotheses framework i.e. superiority, equivalence or noninferiority hypothesis testing and which group comparisons will be analysed	H0 There is no significant difference in the expression of molecules among children with varying effects of myopia control. H1 There is a significant difference in the expression of molecules among children with varying effects of myopia control.
3.5	Statistical interim analyses and stopping guidelines (if applicable) Describe how and when interim analyses will be performed, and potential planned adjustment of significance level due to interim analyses. Describe guidelines for stopping the trial early.	The ProMOC study is an exploratory cross-sectional study that does not involve interim analyses or predefined stopping guidelines.
3.6	Timing of outcome assessments and follow-up Describe time points at which outcomes/covariates will be measured (consider a figure to visualize the time windows of measurements – see appendix)	The study consists of a single examination day. Label-free quantification nano liquid chromatography tandem mass spectrometry (LFQ nLC-MS/MS) will be used to identify and quantify differences in protein levels among children using ortho-k lenses with varying levels of myopia control effectiveness.
3.7	Timing of final analysis i.e. all outcomes analysed collectively or analyses performed according to planned follow-ups	All outcomes are analyzed collectively.

Section 4: Statistical principles and protocol deviations

4.1	Confidence intervals and P-values Specification of level of statistical significance and confidence intervals to be reported. Describe, if relevant, rationale for adjustment for multipel testing and how type 1 error will be controlled for	The level of statistical significance for this study is set at $p < 0.05$. Confidence intervals will be reported at the 95% confidence level to estimate the precision of the results. To control for type I error due to multiple testing, corrections will be applied using a permutation-based method with a false discovery rate (FDR) set at 0.05.
4.2	Adherence/compliance and protocol deviations Define adherence/compliance and how this is assessed in the	In this study, adherence refers to participants wearing ortho-k lenses as instructed prior to lens collection. Compliance was assessed based on participant confirmation and the examination of lens wear on the day of collection.

	study. Define protocol deviations and which protocol deviations will be summarized and presented	Protocol deviations could include: - Failure to wear the ortho-k lenses as required prior to sample collection. - Delays or errors in sample collection, handling, or processing. - Participants who do not meet the inclusion criteria or exhibit exclusion criteria at the time of sample collection.
4.3	Analysis populations Define analysis population i.e. intention-to-treat, per-protocol, complete case, safety population	As no adherence challenges are expected in this study, the intention-to-treat (ITT) and per-protocol (PP) analysis populations are anticipated to yield identical results. Therefore, a single analysis will be performed using all available data, without the need for distinction.

Section 5: Study population

5.1	Screening (if applicable) Describe screening data to determine eligibility (i.e. scoring and scales)	Eligibility for participation was determined through a screening process at the Department of Ophthalmology, Vejle Hospital, based on the study's inclusion and exclusion criteria.
5.1	Eligibility Summarize in- and exclusion criteria	Inclusion criteria - Children aged 5 to 14 receiving only ortho-k treatment. - Children at or above the 95th percentile of myopia growth curves for European children at the start of treatment. - Ortho-k treatment and follow-up duration of at least 6 months. Exclusion criteria - Current use of eye drops or medications that may affect pupil size, accommodation, or impact the ocular surface. - Objective signs of dry eyes on slit-lamp examination, defined as an Efron score for corneal staining higher than grade 2. - Active eye infection. The exclusion criteria have been clarified further to enhance transparency.
5.2	Recruitment and flow chart Specification of steps in the recruitment process i.e. enrollment, screening allocation for use in flow chart (see appendix)	Children treated with ortho-k at the Department of Ophthalmology, Vejle Hospital, University Hospital of Southern Denmark, were invited to participate in the study. The children were referred from private ophthalmic practices based on cycloplegic spherical equivalent refractive error (cSER) in relation to age.

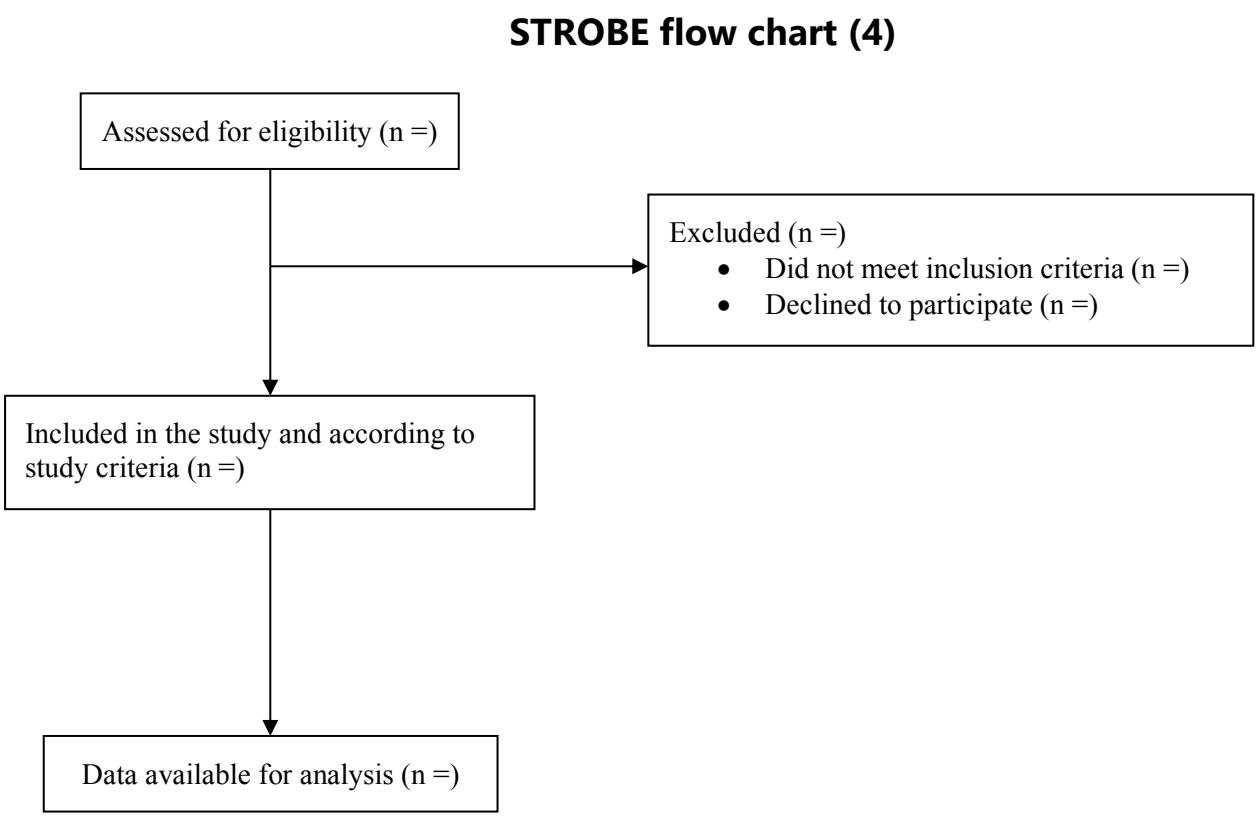
5.3	Withdrawal/loss to follow-up Specification on how reason and timing of withdrawal or loss to follow-up will be recorded and presented (i.e. in the flow chart – see appendix)	Reasons for withdrawal or loss to follow-up will be recorded and categorized as follows: - Failure to meet inclusion criteria. - Declined participation.
5.4	Baseline patient characteristics List of baseline characteristics and how these data will be descriptively summarized in a "Table 1" (see appendix)	Normal distribution: mean, SD Not normally distributed: median, range (As determined by a normal quantile-quantile plot) Age: years Gender: male/female (fraction, %) Ortho-k treatment duration: months Cycloplegic Spherical Equivalent Refractive Error (cSER): before ortho-k treatment initiation Axial length (AL): - before ortho-k treatment initiation - AL progression during the 6 months prior to ortho-k collection Other relevant data: such as compliance with ortho-k lens usage

Section 6: Analysis

6.1	Exposure and outcome definitions Describe details on exposure i.e. assessment, definitions, units and thresholds or the intervention/treatment under study. List and describe details on primary and secondary outcomes i.e. definition of outcome and timing, specific clinical measurements and units (i.e. mmol/mol) or any calculation or transformation of data to derive the outcome (i.e. sum score, change from baseline, logarithm, quality-of-life scoring algorithm)	The primary outcome is the difference in protein levels among children using ortho-k lenses with varying levels of myopia control effectiveness. Protein levels will be measured using mass spectrometry label-free quantification (LFQ), which will identify and quantify the proteins. We have defined myopia control effectiveness as axial length progression during six months prior to ortho-k collection, measured by subtracting the AL ≤ 35 days from ortho-k collection from the AL ≥ 6 months before ortho-k collection. The result will be adjusted to ensure the progression reflects a six-month period.
6.2	Primary analysis methods Describe in details which statistical methods will be used (i.e. regression), how treatment effects will be presented (i.e. which effect	The primary analysis will involve a mixed model to compare protein levels identified and quantified using mass spectrometry label-free quantification (LFQ). The treatment effect will be presented as the mean difference in protein levels between

	measure - OR, HR etc.) and if estimates will be adjusted for covariates (see appendix). If analyses will be adjusted for covariates, describe how the sufficient adjustment set will be defined (i.e. using DAGs) Describe methods used to check assumptions (i.e. normality, proportional hazards) behind the statistical models, and alternative methods if assumptions about distribution do not hold.	groups, along with 95% confidence intervals and corresponding p-values. Since this is an exploratory cross-sectional study, the primary analysis will not include adjustments for covariates. Assumptions of normality for protein level distributions will be assessed using normal quantile-quantile (Q-Q) plots. Levene's test will be used to assess the homogeneity of variance. To control for type I error, a false discovery rate (FDR) adjustment will be performed using the permutation-based method in Perseus software, with the number of randomizations set to 250 and an S0 parameter of 0.1.
6.3	Additional analysis methods Describe any planned sensitivity and subgroup analysis including how subgroups will be defined (see appendix).	No additional analyses will be performed.
6.4	Missing data Describe how missing data will be explored and which assumptions and methods will be used to handle missing data (i.e. multiple imputation)	Missing data are assumed to be missing at random (MAR) and will be handled using mixed effects regression analysis.
6.5	Harms (only applicable in experimental studies) Describe the collection of safety data i.e. data on severity, expectedness, causality. Describe grouping and analyses planned i.e. incidence analyses on grade 3-4 events only.	Since this is a study without intervention, there are no risks, side effects, or disadvantages associated with participation for the subjects.
6.6	Statistical software Specify statistical packages to be used for the analyses	The statistical analyses will be performed using Stata.

5.2-3 Flow chart template for observational studies



5.4 Baseline table ("Table 1") template

Table 1: Characteristics of the study population

	All subjects Mean ± SD Median (range) (n = xx)
Age, years	xx ± xx
Sex, n (M%)	xx (xx%)
OK treatment duration, months	xx ± xx
SER cyclo, D	xx (xx-xx)
AL before OK treatment initiation, mm	xx ± xx
AL progression during the 6 months prior to OK collection, mm	xx ± xx

AL = axial length; D = diopter; F = female; M = male; mm = millimeters; n = number; OK = orthokeratology, ortho-k; SD = standard deviation; SER cyclo = cycloplegic spherical equivalent refractive error.