# Study Protocol and Statistical Analysis Plan

*ClinicalTrials.gov Results Reporting Document*

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| **Official Title** | Blood Leukocyte Profiling in Eosinophilic Type 2 Asthma: Influence of Systemic IL-5 Targeting |
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**Purpose of this document.** This English protocol and statistical analysis plan summary was prepared for the ClinicalTrials.gov study document requirement at results reporting. It is based on the Swedish ethics-approved research plan and summarizes the mepolizumab-focused observational analysis reported under NCT05689931. It is not a manuscript, journal supplement, or participant information sheet.

**Confidentiality note.** This document contains no names or identifiers of research participants. Results and participant information are described only at study-group level.

## 1. Background and Rationale

Asthma is a heterogeneous inflammatory airway disease. A subset of patients have moderate-to-severe or severe asthma despite inhaled corticosteroids and bronchodilator therapy. Biological antibody treatments targeting type 2 inflammatory pathways are used in selected patients, but detailed immunological profiles before and after such treatment remain incompletely characterized.

The approved research plan proposed prospective observational immune profiling in asthma patients starting or already receiving biological treatment, in asthma patients not eligible for such treatment, and in healthy controls. The ClinicalTrials.gov record NCT05689931 concerns the mepolizumab-focused component of this program, with emphasis on blood leukocyte profiling in eosinophilic type 2 asthma and the influence of systemic IL-5 targeting.

The study was observational. Mepolizumab treatment was prescribed according to routine clinical criteria and not assigned by the research protocol. Study procedures consisted primarily of additional sampling and assessments for research purposes.

## 2. Objectives and Scientific Questions

The overall objective was to characterize systemic immune-cell profiles in patients with moderate-to-severe/severe asthma and to relate these profiles to clinical features such as allergic sensitization, asthma symptoms, lung function, medication, and soluble biomarkers.

A secondary objective was to evaluate how antibody-based biological treatment, specifically IL-5 neutralization with mepolizumab in this ClinicalTrials.gov record, was associated with changes in peripheral blood leukocyte phenotypes.

The main scientific question for this record was whether detailed profiling of peripheral blood leukocytes, including type 2-related immune-cell readouts, could capture baseline immunophenotypes and exploratory changes after initiation or maintenance dosing of mepolizumab.

## 3. Study Design

This was a prospective observational cohort study conducted in Sweden. Participants were allocated to observational groups according to clinical status and treatment status. There was no randomization and no protocol-driven assignment of treatment.

The approved research program included asthma patients starting biological treatment, asthma patients already on biological treatment, asthma patients not eligible for biological treatment, and healthy controls. For NCT05689931, the implemented analysis focused on mepolizumab-treated type 2-high asthma and comparator groups.

| Group | Description | Planned/role in protocol | Sampling schedule for this record |
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| Group 1 | T2-high asthma patients initiating mepolizumab | Longitudinal initiation cohort | Baseline before treatment, Day 2, Week 1, Week 4, Month 4 |
| Group 2 | T2-high asthma patients on maintenance mepolizumab | Maintenance/refill cohort | Pre-refill and approximately 1 week post-refill |
| Group 3 | Severe asthma patients not eligible for biologics | Asthma comparator group | Baseline only |
| Group 4 | Healthy controls | Non-asthma comparator group | Baseline only |

## 4. Study Population

Eligible asthma participants were adults with moderate-to-severe/severe asthma followed at the Department of Allergology and Lung Medicine, Skåne University Hospital, Lund. Healthy controls were recruited as non-asthma comparator participants.

Inclusion categories were: patients starting biological antibody treatment according to routine clinical criteria; patients already receiving biological antibody treatment; severe asthma patients not judged eligible for biological treatment; and healthy controls without known asthma, allergy, other lung disease, or immune disease.

Key exclusion criteria in the approved research plan included recent respiratory infection within the preceding 3 weeks, pregnancy, and breastfeeding. All participants were adults (>18 years).

The research plan described an exploratory design; therefore, formal sample-size calculations were not considered feasible. The planned enrollment in the ClinicalTrials.gov record was larger than the final analyzed cohort. Actual enrollment and longitudinal follow-up were substantially limited because the study started shortly before the COVID-19 pandemic, which restricted the capacity and feasibility of repeated clinical research visits at the lung clinic. After the pandemic, there was a lag phase before clinical research activity could resume fully. During this period, clinical treatment practice also changed, with mepolizumab used less frequently as other biologics for type 2-high asthma became more commonly used. These factors reduced the number of eligible patients initiating mepolizumab during the study period and resulted in a small longitudinal initiation cohort.

## 5. Study Procedures and Data Collection

After informed consent, participants were assigned a study code. Clinical information was recorded in case report forms, including age, sex, asthma history, allergic sensitization, smoking status, comorbidities, medication, lung function, asthma symptoms, and quality-of-life/symptom questionnaire data.

Study assessments included blood sampling, asthma-related questionnaires, fractional exhaled nitric oxide (FeNO), spirometry, and, in the broader approved plan, sputum induction and skin capillary/microneedle sampling. For the mepolizumab-focused ClinicalTrials.gov record, the reported results center on blood leukocyte profiling and selected clinical variables.

Blood samples were used for routine total leukocyte counts and differential counts, preparation of formalin-fixed paraffin-embedded blood leukocyte pellets, and biomarker-related analyses. The blood leukocyte pellet method enabled histology-based immune-cell profiling by immunohistochemistry/immunofluorescence and computerized single-cell image analysis.

Data were handled in coded form. The code key was stored securely and separately, accessible only to authorized members of the research group. Data were to be retained according to the ethics-approved research plan.

## 6. Laboratory and Imaging Methods

Peripheral blood leukocytes were enriched and processed into formalin-fixed paraffin-embedded (FFPE) blood leukocyte pellet sections. Sections were stained using immunohistochemical and immunofluorescence marker panels and digitized using whole-slide scanning.

Marker panels included pro-major basic protein (proMBP) and eosinophil cationic protein (ECP) for eosinophil-lineage and basophil-related readouts; CD4, GATA3, and T-bet for CD4 T-helper axis readouts; CD19, CD20, and HLA-DR for B-cell readouts; and CD45, CD3, and CD56 for broader leukocyte/T-cell/NK-cell profiling where relevant.

Computerized image analysis was used to segment nucleated cells and quantify marker-positive cell populations at single-cell level. Readouts were reported as cell counts, proportions, ratios, or marker-defined subpopulation frequencies, depending on the marker panel and analysis question.

## 7. Outcome Measures and Readouts

The ClinicalTrials.gov outcomes were specified as changes in blood eosinophil marker expression, blood basophil marker expression, blood leukocyte composition, T-lymphocyte marker expression, B-lymphocyte marker expression, total leukocyte counts, spirometry variables, and FeNO.

For results reporting, the broad leukocyte outcome headings were mapped to the actual measured marker-defined readouts from the final analysis. Representative longitudinal readouts in the mepolizumab initiation cohort included low-intensity proMBP-positive eosinophil-lineage proxy cells, high-intensity proMBP-positive basophil-like cells, low-/mid-intensity proMBP-positive cells as a proportion of the proMBP panel, log2(GATA3/T-bet) CD4 Th-axis balance, and HLA-DR positivity within the CD19/CD20-gated B-cell population.

The primary longitudinal timepoints used for concise registry reporting were baseline, Day 2, and Month 4 after mepolizumab initiation. Additional timepoints were included in the research analysis where available.

Implementation note for results reporting: Because only Group 1 comprised patients initiating mepolizumab with repeated post-initiation sampling, the longitudinal change-from-baseline leukocyte outcomes in the ClinicalTrials.gov Results Section are reported for Group 1 only. Groups 2-4 were used for comparator or baseline purposes and were not sampled longitudinally after mepolizumab initiation. The resulting registry outcome tables should therefore be interpreted as descriptive summaries of the implemented exploratory analysis rather than as confirmatory efficacy analyses.

## 8. Statistical Analysis Plan

The study was exploratory. Analyses were conducted descriptively and inferentially according to the comparison being addressed. Because of the small sample size, results were interpreted as hypothesis-generating rather than confirmatory.

Baseline group comparisons used Kruskal-Wallis tests across relevant groups. When overall tests were significant, prespecified pairwise comparisons were performed using two-sided Mann-Whitney U tests with Holm adjustment for pairwise comparisons. False discovery rate (FDR) q-values were computed across endpoint panels using the Benjamini-Hochberg procedure.

Maintenance/refill comparisons in participants already receiving mepolizumab used paired pre- versus post-refill comparisons with two-sided Wilcoxon signed-rank tests. FDR q-values were calculated across endpoint panels.

Longitudinal initiation analyses in participants starting mepolizumab used Friedman tests for within-subject differences across visits where complete-case data were available. Prespecified baseline-to-follow-up comparisons used two-sided Wilcoxon signed-rank tests with Holm adjustment within each endpoint. Given the small sample size, effect sizes, ranges, and directional consistency were emphasized.

For ClinicalTrials.gov results reporting, selected representative outcomes were reported with number analyzed, mean, and full range for each timepoint. Formal statistical analyses were not added to each registry outcome table unless required, to maintain a concise descriptive presentation consistent with the exploratory nature of the study.

## 9. Ethics, Consent, and Safety Considerations

The study was approved by the Swedish ethics review authority/regional ethics review process and was conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants before participation.

Risks were considered low. Additional study procedures included blood sampling, questionnaires, FeNO measurement, sputum induction, and skin capillary/microneedle sampling in the broader approved protocol. Possible discomfort included venipuncture discomfort or minor hematoma, mild discomfort from skin sampling, and transient symptoms during sputum induction. Sputum induction was performed with clinical precautions including bronchodilator pre-treatment and lung-function monitoring.

Biological treatment was given according to clinical practice and not assigned by the research protocol. Research results were not used for clinical decision-making. Participant data were coded, and any public reporting was to be performed at group level only.

## 10. Reporting, Funding, and Data Access

Results were planned to be reported at scientific meetings and in peer-reviewed international scientific journals after completion of analyses. ClinicalTrials.gov results are provided as structured registry results and do not replace a scientific manuscript.

Data processing and written reporting were the responsibility of the research group and responsible investigators. Data were collected in case report forms and stored in coded, access-controlled files.

Funding sources included Swedish research grants and independent funding from GlaxoSmithKline for analysis-related work. No personal financial interests of the responsible investigators were specified in the ethics-approved research plan.

## 11. Document Notes for ClinicalTrials.gov

This document is an English summary of the study protocol and statistical analysis plan prepared for ClinicalTrials.gov results reporting. The original ethics submission was prepared in Swedish and covered a broader severe-asthma immune-profiling research program. This document is tailored to the mepolizumab-focused record NCT05689931.

The document intentionally excludes participant identifiers, manuscript-style figures, journal discussion, and detailed result interpretation. Structured results are provided in the ClinicalTrials.gov Results Section.

Recruitment limitation note: The reduced final sample size reflects pandemic-related restrictions, post-pandemic delays in restarting clinical research workflows, and subsequent changes in severe asthma biologic prescribing patterns. These constraints are reported in the ClinicalTrials.gov Participant Flow and Limitations/Caveats fields to clarify why the final analyzed mepolizumab-initiation cohort was smaller than originally planned.