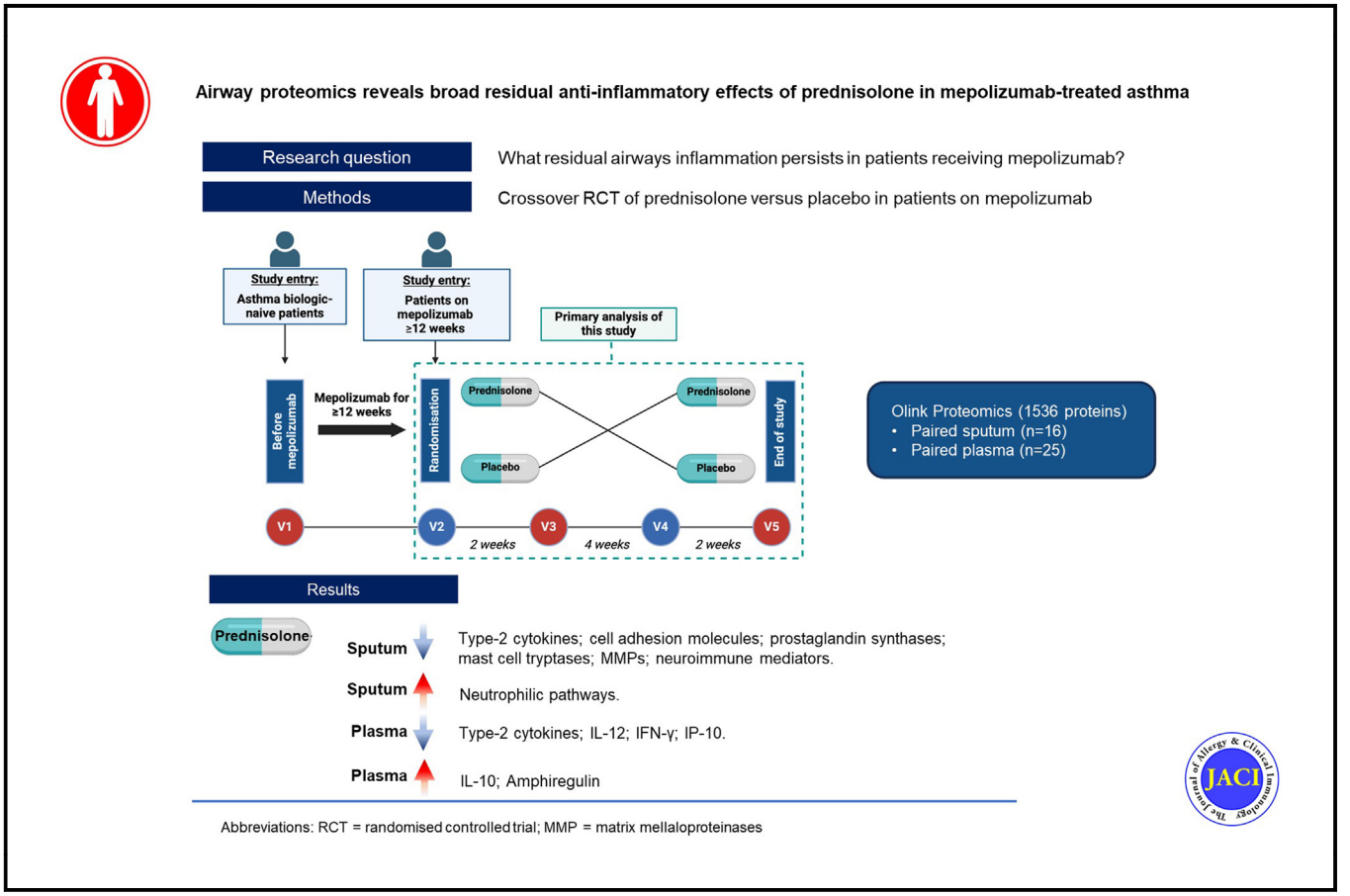
Airway proteomics reveals broad residual anti-inflammatory effects of prednisolone in mepolizumab-treated asthma

Imran Howell, MBBCh, Freda Yang, MD, Vanessa Brown, PhD, Jennifer Cane, PhD, Emanuele Marchi, PhD, Adnan Azim, PhD, et al

GRAPHICAL ABSTRACT



Capsule summary: Proteomic analysis of airway samples from a placebo-controlled, crossover trial showed that prednisolone has extensive anti-inflammatory airway effects on top of mepolizumab in some individuals. These effects may be clinically relevant in residual exacerbations.

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Airway proteomics reveals broad residual anti-inflammatory effects of prednisolone in mepolizumab-treated asthma

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Background: Mepolizumab is an anti-IL-5 mAb treatment for severe eosinophilic asthma that reduces asthma exacerbations. Residual airway inflammation with mepolizumab therapy may lead to persistent exacerbations. Oral corticosteroids remain the main treatment for these residual exacerbations.

Objective: Our study aimed to explore the corticosteroid responsiveness of airway inflammation after mepolizumab treatment to find potentially treatable inflammatory mechanisms beyond the IL-5 pathway.

Methods: The MAPLE trial was a multicenter, randomized, double-blind, placebo-controlled, crossover study of 2 weeks of high-dose oral prednisolone treatment at stable state in 27 patients treated with mepolizumab for severe eosinophilic asthma. We analyzed paired sputum (n = 16) and plasma (n = 25) samples from the MAPLE trial using high-throughput Olink proteomics. We analyzed additional sputum proteins using ELISA.

Results: In patients receiving mepolizumab, prednisolone significantly downregulated sputum proteins related to type 2 inflammation and chemotaxis including IL-4, IL-5, IL-13, CCL24, CCL26, EDN, CCL17, CCL22, OX40 receptor, FCER2, and the ST2 receptor. Prednisolone also downregulated cell adhesion molecules, prostaglandin synthases, mast cell tryptases, MMP1, MMP12, and neuroimmune mediators. Neutrophilic pathways were upregulated. Type 2 proteins were also downregulated in plasma, combined with IL-12, IFN-γ, and IP-10. IL-10 and amphiregulin were upregulated.

Conclusions: At stable state, prednisolone has broad anti-inflammatory effects on top of mepolizumab. These effects are heterogeneous and may be clinically relevant in residual exacerbations. (J Allergy Clin Immunol 2024;154:1146-58.)

Key words: *Asthma, proteomics, clinical trial, inflammation, corticosteroids, biologics*

Abbreviations used
BH: Benjamini-Hochberg
ECM: Extracellular matrix
FDR: False discovery rate
FENO: Fraction exhaled nitric oxide
MAPK: Mitogen-activated protein kinase
NF-κB: Nuclear factor-κB
NPX: Normalized protein expression
OCS: Oral corticosteroids

Oral corticosteroids (OCS) are a widely prescribed treatment for asthma exacerbations.[1] Clinical evidence for use of OCS at exacerbation is based on trials demonstrating a reduction in relapse and accelerated symptom resolution.[2] Biologically, OCS reduce eosinophilic inflammation, evidenced by a rapid decline in blood eosinophils through apoptosis and inhibition of IL-5–induced production by the bone marrow.[3,4] However, OCS have broader anti-inflammatory effects that may also be clinically important. The placebo-controlled BIOAIR study identified inflammatory proteins downregulated by prednisolone in both mild/moderate and severe asthma at stable state in plasma.[5] These included chemokines and proteins involved in recruiting eosinophils to the airways.[6] However, anti-inflammatory effects of OCS also extend to the airway mucosa. A placebo-controlled human bronchoscopy study in patients with stable, moderate asthma found that prednisolone reduced airway eosinophilia, tryptase single-positive mast cells, IL-4, IL-5, and $CD3^+$ T lymphocytes.[7]

Systemic mAb therapy has revolutionized the treatment of severe eosinophilic asthma. Placebo-controlled trials of mepolizumab, an anti-IL-5 mAb, demonstrated approximately a 50% reduction in the yearly exacerbation rate and maintenance dose of OCS.[8] This is important because repeated exposure to OCS is associated with serious acute and chronic adverse effects.[9] However, some patients continue to experience asthma exacerbations despite mepolizumab therapy. The MEX study showed that roughly half of these events had high sputum eosinophils, which suggests that inflammatory pathways beyond IL-5 may remain upregulated and clinically relevant in some people.[10] The nature of these pathways is not understood.

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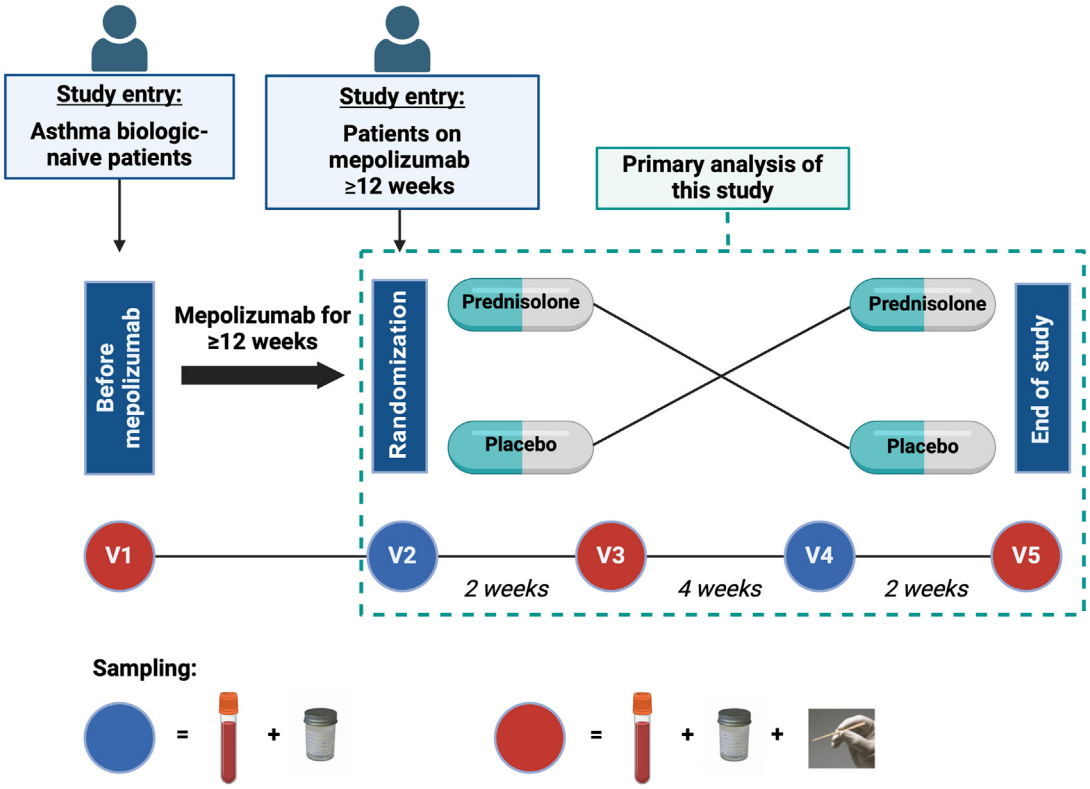


FIG 1. Schematic of the MAPLE study design and sampling. Participants with severe asthma entered the study at either visit 1 (before mepolizumab) or visit 2 (at least 12 weeks after mepolizumab initiation). The primary analysis was a crossover study of prednisolone vs placebo at stable state in participants established on mepolizumab. At visit 2, all participants were randomized to either prednisolone or placebo treatment for 2 weeks, followed by a 4-week washout, and at visit 4 crossed over to receive the opposite treatment for 2 weeks. At visits 1, 3, and 5 *(red circle)*, participants had blood, induced sputum, and nasal scrape sampling. At visits 2 and 4 *(blue circle)*, participants had blood and induced sputum sampling. Blood sampling consisted of EDTA and serum separator tube blood tubes. Samples at visit 2 occurred after at least 12 weeks of continuous mepolizumab treatment.

The MAPLE trial was an exploratory, multicenter, randomized, double-blind, placebo-controlled, crossover study of prednisolone in adults treated with mepolizumab at stable state. The research question was to understand whether prednisolone had clinical and inflammatory effects in mepolizumab-treated participants at stable state. The aims were to elucidate steroid-responsive inflammatory asthma mechanisms beyond the IL-5 pathway and inform the rationale for withholding prednisolone in mepolizumab-treated individuals at exacerbation. There was no significant clinical improvement with prednisolone in MAPLE.[11] In this article, we report the planned proteomic analysis of sputum and plasma samples from the MAPLE trial to test the hypothesis that prednisolone exerts additional anti-inflammatory effects on top of mepolizumab at stable state.

METHODS

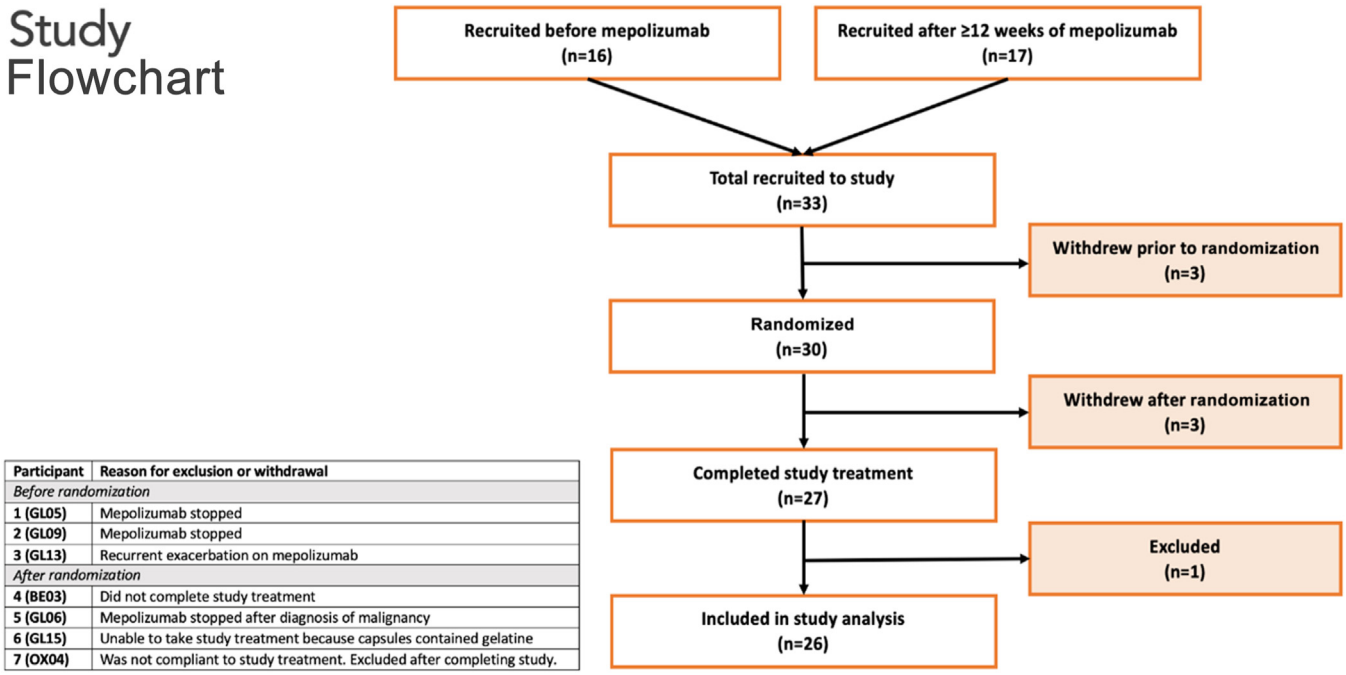
Study design

The MAPLE study population, design, and ethical approval were described in detail previously.[11] This study is registered on Clinicaltrials.gov (NCT03610685). Briefly, participants were recruited before or after starting mepolizumab for usual clinical care. All participants provided written informed consent before any study-specific procedures took place. All participants received at least 12 weeks of mepolizumab and were OCS-free for more than 4 weeks before they were randomly assigned (1:1) to prednisolone (0.5 mg/kg/day, maximum dose of 40 mg/day, 14 ± 2 days) followed by a 4-week washout period and then matched placebo or *vice versa* (Fig 1). All mepolizumab injections were administered by clinical staff, and all study participants were adherent to 4 weekly (± 2 days) mepolizumab injections for the duration of the study. Prednisolone adherence was confirmed with tablet counts and serum prednisolone and cortisol levels.

A crossover design was appropriate for our research question because it uses within-patient sampling to minimize biological variability. Because the trial was performed in patients with stable asthma, the treatment durations of prednisolone and placebo were short, and the risk of period effects was low. The elimination half-life of prednisolone is 4 hours, so we minimized the risk of carryover effects by using a conservative 28-day washout.

Sputum and blood sampling and processing

Fig 1 describes the trial sampling. Sputum was induced using hypertonic saline (see this article's Methods section in the Online Repository at www.jacionline.org). Samples were transferred to the laboratory within 2 hours for initial processing. Sputum plugs were selected from saliva, weighed, and centrifuged in PBS at 790*g* for 10 minutes at 4°C. Samples were aliquoted and stored at −80°C in



Participant	Reason for exclusion or withdrawal
Before randomization	
1 (GL05)	Mepolizumab stopped
2 (GL09)	Mepolizumab stopped
3 (GL13)	Recurrent exacerbation on mepolizumab
After randomization	
4 (BE03)	Did not complete study treatment
5 (GL06)	Mepolizumab stopped after diagnosis of malignancy
6 (GL15)	Unable to take study treatment because capsules contained gelatine
7 (OX04)	Was not compliant to study treatment. Excluded after completing study.

FIG 2. Study flowchart. Comparison between prednisolone and placebo.

cryovials until further processing. Blood collected in EDTA tubes was centrifuged at 1500*g* ± 200*g* for 15 minutes, and plasma was aliquoted and stored at −80°C in cryovials until further processing.

Olink proteomic analysis of sputum and plasma samples

Sputum supernatant and plasma samples from all visits were analyzed by Olink Explore 1536 (Olink Proteomics, Uppsala, Sweden) proximity extension assays for 1536 proteins. Olink hybridizes DNA oligonucleotide-labeled antibody pairs to proteins, extends oligonucleotides by DNA polymerase, quantifies them with next-generation sequencing, and log2 transforms concentrations to a normalized protein expression (NPX) value. NPX is a relative quantification measure.

Statistical analyses were performed with the Olink Analyze package in RStudio version 4.2.2 (https://cran.rstudio.com/bin/windows/base/old/4.2.2/), according to the manufacturer's instructions. We determined the effect of prednisolone versus placebo on paired sputum and plasma proteins using a change from baseline analysis in a linear mixed effects model.[12] The type and sequence of treatment were fixed effects, and participant identification was the random effect, with Benjamini-Hochberg (BH) correction for multiplicity of testing. A false discovery rate (FDR) <0.05 and absolute log2 fold change >0.5 was considered significant. The effect of mepolizumab on sputum and serum proteins was determined using paired *t* tests comparing samples from visit 1 with visit 2 with BH correction.

Unsupervised pathway analysis was performed using clusterProfiler (https://www.bioconductor.org/packages/release/bioc/html/clusterProfiler.html), with significant proteins as the query dataset against the full proteome dataset. We created heatmaps with hierarchical clustering by Euclidean distance using pheatmap (https://www.rdocumentation.org/packages/pheatmap/versions/1.0.12). We constructed a correlation matrix of Pearson correlation coefficients with body mass index, pre-prednisolone fraction exhaled nitric oxide (FENO), pre-prednisolone sputum eosinophil and neutrophil percentages, and change in FEV_1 (L), change in FENO (ppb), and change in protein expression for biologically important sputum proteins from before to after prednisolone using corrplot (https://www.rdocumentation.org/packages/corrplot/versions/0.94/topics/corrplot). We assessed Spearman correlation coefficients between 30 sputum and plasma Olink proteins chosen because of their importance in asthma. We also assessed intraclass correlation coefficients of all Olink proteins in sputum and plasma in participants treated with mepolizumab using the pre-prednisolone, pre-placebo, and post-placebo samples.

ELISA of sputum samples

Sputum supernatants from all visits were assayed using the Ella ELISA platform (Bio-Techne, Minneapolis, Minn) for 15 analytes. ELISA was performed for absolute quantification of a panel of proteins of interest, either because they were unavailable on Olink Explore or because they were could not be quantified absolutely. These proteins were IL-17E/IL-25, IL-1α, IL-1β, IL-2Ra, IL-33, IL-6, IL-6R, MPO, IL-3, YKL-40, IL-1RA, GM-CSF, TSLP, EDN, and IL1R1. The effect of prednisolone versus placebo was analyzed on paired samples using linear mixed effects models, and the effect of mepolizumab was analyzed using paired *t* tests, with BH correction (FDR <0.05).

Sample size

As this was a secondary analysis on a trial powered on clinical end points, a power calculation was not performed. Findings are exploratory and descriptive.

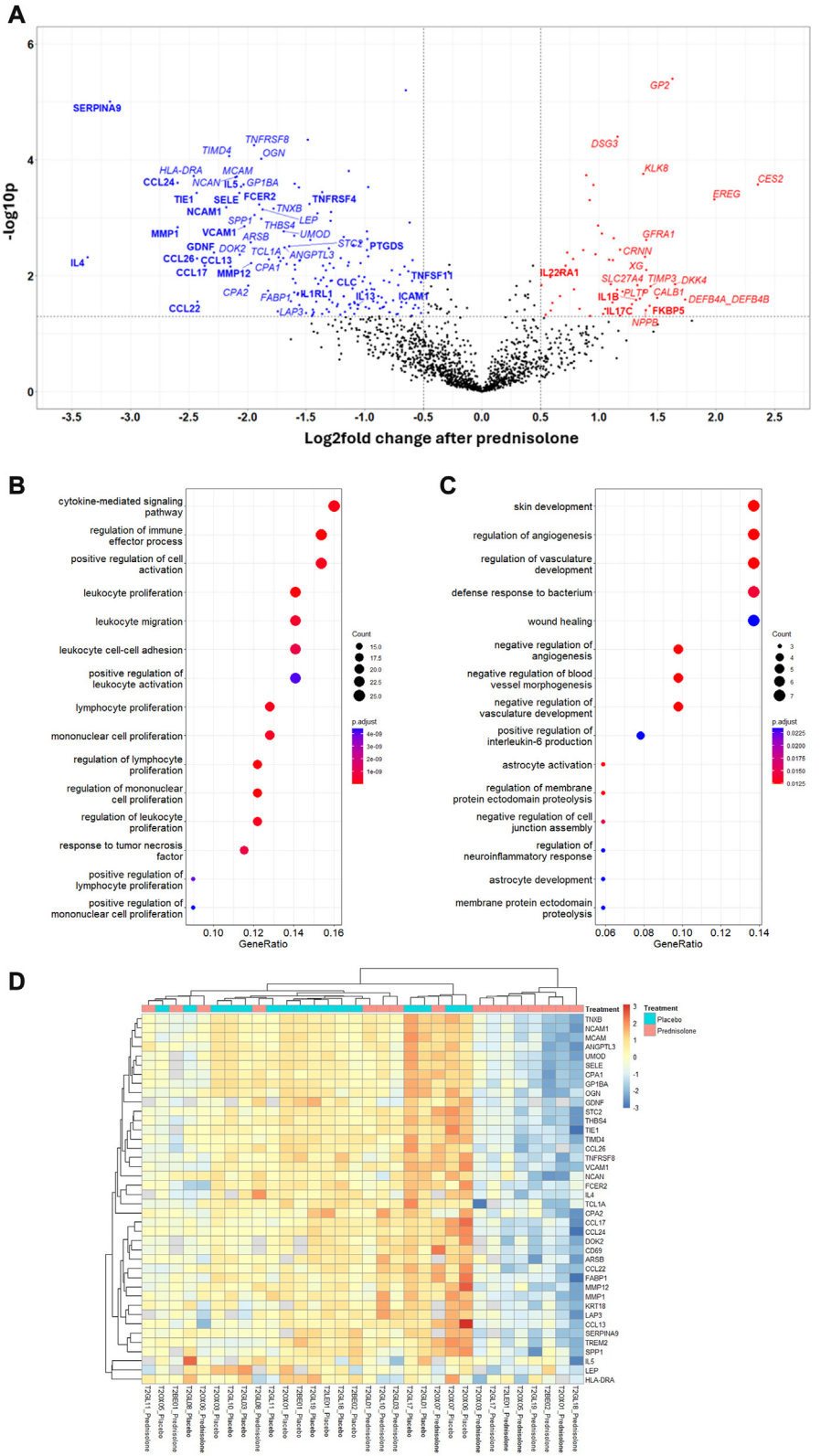


FIG 3. Summary of prednisolone effects on proteins measured by Olink in sputum. **(A)** Volcano plot of differentially expressed proteins by prednisolone in sputum. An absolute log2 fold >0.5 *(vertical line)* and FDR <0.05 *(horizonal line)* were considered significant. *Blue* denotes downregulated by prednisolone; *red*, upregulated by prednisolone; *black*, nonsignificant. **(B** and **C)** Top 15 most overrepresented Gene Ontology biological processes significantly downregulated *(B)* or upregulated *(C)* by prednisolone in sputum. *Color* denotes adjusted *P* value (FDR <0.05). **(D)** Heatmap showing individual change in NPX (n = 16 participants) after prednisolone and placebo in the 40 most significantly downregulated sputum proteins from the linear mixed effects model. Participants and proteins underwent unsupervised clustering. *Top row* denotes treatment received, either placebo *(blue)* or prednisolone *(red)*. *Other rows*: *red* denotes increased protein expression, and *blue* denotes reduced expression.

TABLE I. Summary of 25 most downregulated proteins by prednisolone versus placebo in sputum (Olink)

Protein	Log2 fold change	Adjusted *P* value	Function
IL4	−3.37	4.81×10^{-3}	Stimulates proliferation of T_H2 cells, B-cell class switching to IgE, and B-cell differentiation to plasma cells
SERPINA9	−3.18	9.79×10^{-6}	Inhibits trypsin-like serine proteases
CCL24	−2.6	2.47×10^{-4}	Eotaxin-2; induces eosinophil chemotaxis
MMP1	−2.6	1.44×10^{-3}	Breaks down interstitial collagens in ECM
HLA-DRA	−2.46	1.88×10^{-4}	Presents peptides from extracellular proteins; expressed on B lymphocytes, dendritic cells, and macrophages
TIE1	−2.44	3.70×10^{-4}	Cell surface protein that upregulates cell adhesion molecules
CCL26	−2.43	5.02×10^{-3}	Eotaxin-3; induces eosinophil and basophil chemotaxis
CCL22	−2.43	2.79×10^{-2}	Induces T-cell, monocyte, and dendritic cell chemotaxis
CCL17	−2.37	6.68×10^{-3}	Induces T-cell and granulocyte chemotaxis
GDNF	−2.29	3.96×10^{-3}	Promotes neuronal growth and survival
NCAM1	−2.18	6.60×10^{-4}	Mediates neurite outgrowth
TIMD4	−2.16	8.56×10^{-5}	Involved in recognition of apoptotic cells for macrophage phagocytosis
CCL13	−2.15	6.92×10^{-3}	Induces T-cell, monocyte, and granulocyte chemotaxis
NCAN	−2.11	1.99×10^{-4}	Component of ECM and involved in neuronal growth
MCAM	−2.09	1.93×10^{-4}	Vascular endothelial cell adhesion molecule
SELE	−2.07	3.74×10^{-4}	Endothelial adhesion molecule involved in leukocyte recruitment
DOK2	−2.07	4.36×10^{-3}	Scaffolding protein for multimolecular signaling complexes
IL5	−2.06	2.89×10^{-4}	Mediates eosinophil production and activation
GP1BA	−2.04	2.65×10^{-4}	Platelet surface glycoprotein
VCAM1	−2.03	1.40×10^{-3}	Mediates adhesion of lymphocytes, eosinophils, and basophils to the vascular endothelium
CPA2	−2	1.47×10^{-2}	Pancreatic carboxypeptidase
ARSB	−1.97	2.63×10^{-3}	Enzyme that breaks down glycosaminoglycans
MMP12	−1.97	6.09×10^{-3}	Breaks down elastin in ECM
TNFRSF8	−1.95	5.57×10^{-5}	Expressed by T and B cells; leads to activation of NF-κB
SPP1	−1.94	8.87×10^{-4}	Osteopontin; anti-apoptotic factor in macrophages and T cells; also involved in leukocyte chemotaxis

RESULTS

As shown in the study flowchart, 33 patients were enrolled into MAPLE, and 30 participants were randomized (Fig 2). After randomization, 3 participants withdrew: one did not complete the study treatment, one had mepolizumab stopped during the trial after diagnosis of malignancy, and one was unable to take the study treatment because it contained gelatine. After study completion, 1 participant was found to be nonadherent to study treatment according to serum prednisolone and cortisol levels and was excluded. The decision to withdraw these 4 participants after randomization was made before our bioinformatic analysis was conducted, and none of their samples were sent for Olink or ELISA testing.

Olink

Sixteen participants (Tables E1 and E2 in the Online Repository at www.jacionline.org) had paired sputum samples before and after both prednisolone and placebo in the crossover trial for Olink analysis. There were 158 proteins significantly downregulated by prednisolone and 51 proteins significantly upregulated (Fig 3; Table I; Tables E3 and E4 in the Online Repository at www.jacionline.org).

Pathway analysis in sputum showed key downregulated biological processes including chemokine pathways crucial to subepithelial T_H2/type 2 cytotoxic T cell, eosinophil, and mast cell recruitment and pathways related to IL-4 activity. Highly downregulated proteins related to type 2 inflammation and chemotaxis included IL-4, IL-13, IL-5, eotaxins 2 and 3 (CCL24 and CCL26), chemokines CCL17 (TARC) and CCL22, OX40 receptor (TNFRSF4), TIMD4, the low-affinity IgE receptor FCER2, and the ST2 receptor for IL-33 (IL1RL1). Cell adhesion molecules involved in granulocyte trafficking, such as ICAM1, VCAM, TIE1, and e-selectin (SELE), were also downregulated. A reduction in the prostaglandin synthases PTGDS and HPGDS, SIGLEC6, and carboxypeptidase B1 (CPB1) that is closely related to mast cell tryptase (CPA3, not in the Olink panel) suggests a downregulation of mast cell activity. Likewise, mast cell alpha-1/beta-1 tryptase (TPSAB1) was numerically reduced by more than 50%, though nonsignificant (adjusted $P = .07$). Proteins related to nuclear factor-κB (NF-κB) signaling were downregulated, including CD30 (TNFRSF8), CD69, and RANKL (TNFSF11), as were proteins related to tissue remodeling, including MMP12 and MMP1. NCAM1, NCAN, and GDNF, mediators of neuroimmune activity, were also downregulated.

Key upregulated pathways in sputum related to neutrophilic inflammation included IL-1β, IL-17C, and IL-22RA1. FKBP5, a classical hallmark of corticosteroid exposure, was upregulated.

Unsupervised clustering of the proteins with most downregulated expression in sputum by prednisolone versus placebo revealed heterogeneity among individuals (n = 16) in the anti-inflammatory effects of prednisolone (Fig 2, *D*). This heterogeneity was seen also with all proteins with a >0.5 absolute log2 fold change in protein expression (Fig E1 in the Online Repository at www.jacionline.org).

We analyzed the correlation between clinical data and change in protein expression for biologically important sputum proteins after prednisolone (Fig 4). This showed that a reduction in sputum

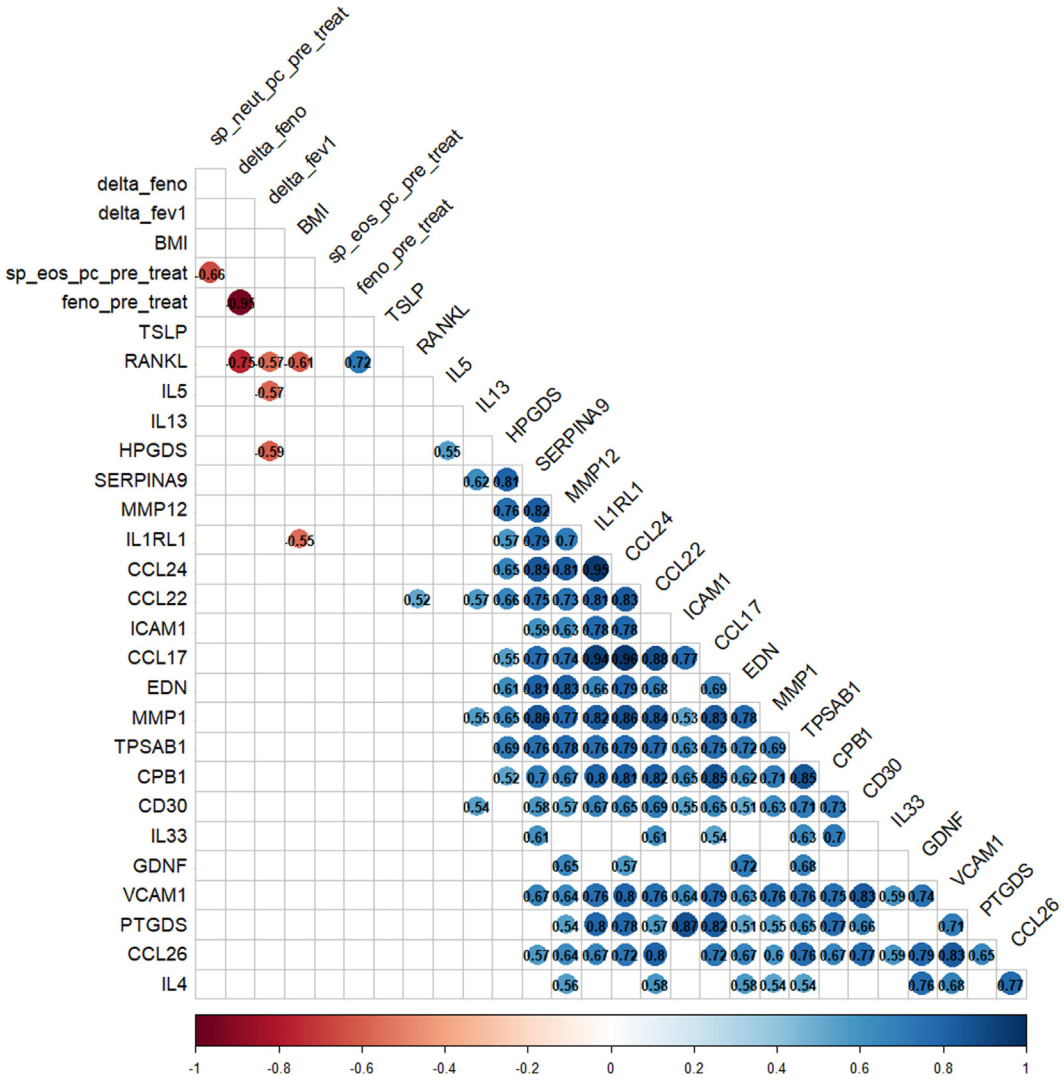


FIG 4. Correlation matrix of sputum proteins and clinical data response to prednisolone. Matrix of Pearson correlation coefficients between clinical data and change in protein expression for biologically important sputum proteins after prednisolone. Only significant positive *(blue)* or negative *(red)* correlations are displayed ($P < .05$). *BMI,* Body mass index at baseline; *delta_feno,* change in FENO with prednisolone; *delta_fev1,* change in FEV_1 (L) with prednisolone; *feno_pre_treat,* FENO before prednisolone treatment; *sp_eos_pc_pre_treatment,* sputum eosinophil percentage before prednisolone treatment; *sp_neut_pc_pre_treatment,* sputum neutrophil percentage before prednisolone treatment.

IL-5 and prostaglandin D synthase had an inverse correlation with FEV_1, change in RANKL was correlated with change in FENO, and IL-33 correlated with the downregulation of other type 2 cytokines and chemokines, but TSLP did not. When assessing Spearman rank correlation between 30 important proteins in asthma, there was significant positive correlation between sputum and plasma eotaxin-3, e-selectin, prostaglandin D synthase, CD30, and amphiregulin (Table E12 in the Online Repository at www.jacionline.org). Intraclass correlation coefficients for Olink sputum proteins of interest generally showed moderate reliability (0.5-0.75) apart from IL-5 and IL-13, which showed poor reliability (<0.5) (Table E13 in the Online Repository at www.jacionline.org).

Twenty-five participants (Tables E1 and E2) had paired plasma samples before and after both prednisolone and placebo in the crossover trial analyzed by Olink. There were 74 proteins significantly downregulated by prednisolone and 53 proteins significantly upregulated (Fig 5; Table II; Tables E5 and E6 in the Online Repository at www.jacionline.org).

The pathway analysis in plasma corresponded to that in sputum, showing that downregulated biological processes included chemokines, type 2 inflammation pathways, and granulocyte trafficking. Relevant downregulated proteins included IL-4, eotaxin-3 (CCL26), CCL17 (TARC), CCL22, CCL19, CD83, OX40 receptor (TNFRSF4), ICAM1, VCAM. Proteins linked to remodeling such as MMP12, MMP1, and MMP10 were reduced. Proteins related to NF-κB signaling were also downregulated, including CD30 (TNFRSF8) and B-cell activating factor (TNFSF13B). There was a decrease in plasma IL-6. Additionally, plasma IL-12 activity and associated plasma IFN-γ and IP-10 (CXCL10) levels were significantly reduced by prednisolone.

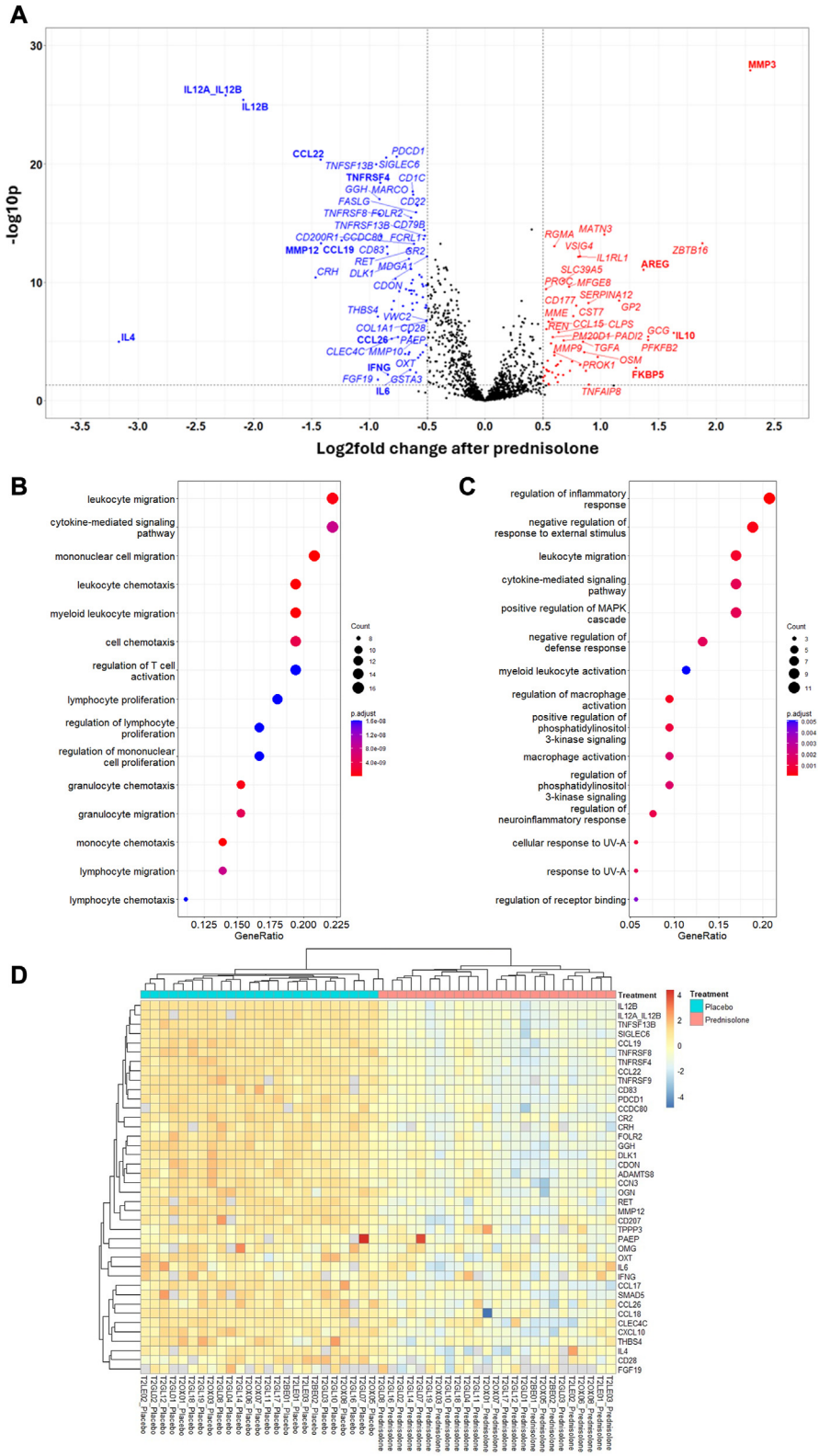


FIG 5. Summary of prednisolone effects on proteins measured by Olink in plasma. **(A)** Volcano plot of differentially expressed proteins by prednisolone in plasma. An absolute log2 fold >0.5 *(vertical line)* and FDR <0.05 *(horizonal line)* were considered significant. *Blue* denotes downregulated by prednisolone; *red*, upregulated by prednisolone; *black*, nonsignificant. **(B** and **C)** Top 15 most overrepresented Gene Ontology biological processes significantly downregulated *(B)* or upregulated *(C)* by prednisolone in plasma. *Color* denotes adjusted *P* value (FDR <0.05). **(D)** Heatmap showing individual change in NPX (n = 25 participants) after prednisolone and placebo in the 40 most significantly downregulated plasma proteins from the linear mixed effects model. Participants and proteins underwent unsupervised clustering. *Top row* denotes treatment received, either placebo *(blue)* or prednisolone *(red)*. *Other rows*: *red* denotes increased protein expression, and *blue* denotes reduced expression.

TABLE II. Summary of 25 most downregulated proteins by prednisolone versus placebo in plasma (Olink)

Protein	Log2 fold change	Adjusted *P* value	Function
IL4	−3.17	1.06×10^{-5}	See Table I
IL12A_IL12B	−2.24	1.55×10^{-26}	Encodes a subunit of IL-12; IL-12 is produced by dendritic cells, macrophages, and neutrophils to promote T_H1 differentiation
IL12B	−2.09	3.88×10^{-26}	Encodes a subunit of IL-12
CRH	−1.46	3.80×10^{-11}	Corticotropin-releasing hormone; stimulates ACTH production
CCL22	−1.42	4.18×10^{-21}	See Table I
MMP12	−1.42	5.07×10^{-14}	See Table I
CCL19	−1.24	2.94×10^{-14}	Involved in T- and B-cell migration to secondary lymphoid organs
TNFSF13B	−0.94	1.03×10^{-20}	B-cell activating factor; important in B-cell proliferation
THBS4	−0.93	7.59×10^{-8}	Adhesive glycoprotein in cell-to-matrix interactions
FGF19	−0.93	1.63×10^{-2}	Regulates bile acid synthesis
GGH	−0.91	9.66×10^{-18}	Key enzyme in intracellular folate homeostasis
TNFRSF8	−0.91	1.67×10^{-16}	See Table I
TNFRSF4	−0.91	3.98×10^{-19}	OX40 receptor; combines with OX40 ligand to prolong T-cell survival, develop memory T cells, and polarize to T_H2 cells
CCDC80	−0.9	1.29×10^{-14}	Promotes cell adhesion and ECM assembly
SIGLEC6	−0.86	2.87×10^{-21}	Glycan-binding cell surface protein highly expressed on mast cells; inhibits mast cell activation after engagement with specific ligands
TNFRSF9	−0.85	9.89×10^{-14}	Cell surface protein that stimulates T- and B-cells and dendritic cells
CD83	−0.84	4.03×10^{-13}	Stabilizes MHC II
IFNG	−0.84	6.50×10^{-3}	IFN-γ; activates macrophages and MHC class II expression
CLEC4C	−0.81	5.93×10^{-6}	Cell surface receptor on dendritic cells involved in antigen capturing
CCL18	−0.81	2.03×10^{-8}	Induces T-cell, B-cell, and dendritic cell chemotaxis
CXCL10	−0.79	3.74×10^{-9}	Induces T-cell, macrophage, and dendritic cell chemotaxis
CCL17	−0.77	4.81×10^{-11}	See Table I
PDCD1	−0.77	2.35×10^{-21}	Cell surface receptor on T and B cells that suppresses T-cell activity
CCL26	−0.75	4.31×10^{-6}	See Table I
CCN3	−0.74	5.80×10^{-10}	Binds to integrin and involved ECM structure and function

Key upregulated processes in plasma included IL-10 and amphiregulin (AREG), important in tissue repair. MMP3 and FKBP5 were increased by prednisolone; both have been previously associated with corticosteroid exposure.[13,14]

Unsupervised clustering of the proteins with most downregulated expression by prednisolone versus placebo in plasma suggested that the anti-inflammatory effects of prednisolone in serum were more homogeneous compared with sputum (n = 25) (Fig 4, *D*). This homogeneity was also seen with all proteins with a >0.5 absolute log2 fold change in protein expression (Fig E2 in the Online Repository at www.jacionline.org).

There was no correlation between change in plasma proteins and FEV_1 after prednisolone. However, change in plasma IL-5, CCL22, and CCL19 was weakly inversely correlated with change in Asthma Control Questionnaire score after prednisolone. Intraclass correlation coefficients for Olink plasma proteins of interest generally showed good to excellent reliability (0.75-1) except for IL-4 and IL-13, which showed poor reliability (<0.5) (Table E13).

ELISA

Sixteen participants provided sputum samples before and after prednisolone and placebo in the crossover trial that underwent ELISA. EDN was significantly reduced after prednisolone (−1180 ng/mL, adjusted $P = .0001$), while IL-1RA was significantly increased (16.9 ng/mL, adjusted $P = 0.045$). All other assays did not significantly change (Fig E3 in the Online Repository at www.jacionline.org; Table E7 in the Online Repository at www.jacionline.org).

Comparison between before and after mepolizumab

Olink. Paired sputum samples from 10 participants were analyzed by Olink before and after starting mepolizumab. There were no significantly changed proteins. To understand the trend in pathways affected by mepolizumab, we performed pathway analysis using FDR <0.15 and absolute log2 fold >0.5. Upregulated pathways included mitogen-activated protein kinase (MAPK) signaling and regulation of leukocyte migration (Fig E5 in the Online Repository at www.jacionline.org).

Paired plasma samples from 10 participants were analyzed by Olink before and after starting mepolizumab. Galectin-10 (CLC) was the only significantly differing protein in plasma, reduced by 70%. Pathway analysis using FDR <0.15 and absolute log2 fold >0.2 showed that mepolizumab initiation downregulated pathways related to lymphoid cell interactions and IL-3, IL-5, and GM-CSF signaling (Fig E6 in the Online Repository at www.jacionline.org).

ELISA. There were no significant differences associated with mepolizumab initiation in any of the sputum proteins tested.

DISCUSSION

Using high-throughput proteomic analysis of airway and blood samples at stable state, we found that oral prednisolone has extensive additional biological effects on top of mepolizumab. Prednisolone markedly reduced type 2 cytokines and chemokines in airway samples (Table I; Fig 6). Eotaxins, CCL17, and CCL22 are released by the airway epithelium and dendritic cells in

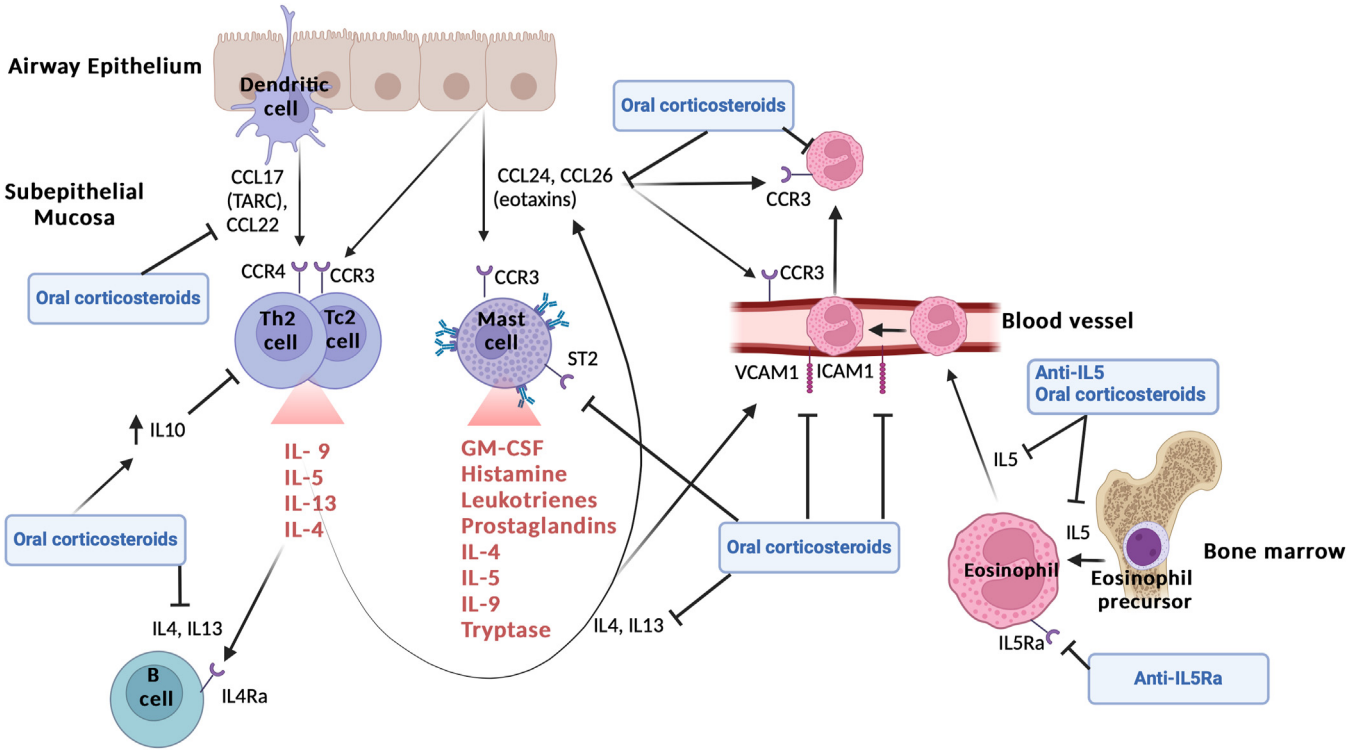


FIG 6. Epithelial effects of OCS in stable asthma on mepolizumab treatment. Eotaxins (CCL24 and CCL26), CCL17 (TARC), and CCL22 are released by the airway epithelium and dendritic cells in response to IL-4 and TSLP. These chemokines have an important role in stimulating T_H2/type 2 cytotoxic T cell and mast cell recruitment via CCR3 and CCR4 receptors. The resultant release of IL-4, IL-5, and IL-13 perpetuates T_H2 cell differentiation and stimulates B-cell maturation and production of IgE. IL-4 also acts in concert with eotaxins, VCAM, ICAM1, E-selectin, and TIE1 to recruit eosinophils, which are produced in the bone marrow after IL-5 stimulation, to the airway epithelium. Mepolizumab blocks IL-5, thereby reducing the production of eosinophils in the bone marrow. OCS have wider effects than mepolizumab, reducing IL-4, IL-13, eotaxins, CCL17, CCL22, and mast cell activity and increasing IL-10 activity, which inhibits T_H2 cell production.

response to IL-4 and TSLP. These chemokines have an important role in stimulating T_H2/type 2 cytotoxic T cell and mast cell recruitment via CCR3 and CCR4 receptors. The resultant release of IL-4, IL-5, and IL-13 perpetuates T_H2 cell differentiation; stimulates B-cell maturation and production of IgE; and acts in concert with eotaxins, VCAM, ICAM1, E-selectin, and TIE1 to recruit eosinophils to the airway epithelium.[8] This inflammation is analogous to an airway epithelial "magnet" recruiting effector granulocytes, analogous to "bombs."[15] Our proteomics data demonstrate that this process remains active and corticosteroid responsive despite mepolizumab treatment, which selectively inhibits recruitment of eosinophils through IL-5. These findings are supported by the reduction of EDN in sputum and *CXCL2*, *CXCL3*, and *CXCL5* transcriptional suppression in nasal tissue (see this article's Results section in the Online Repository at www.jacionline.org).

Biological mechanisms underpinning these effects are complex. Corticosteroids impair dendritic cell maturation; downregulate IL-4 receptor expression; inhibit IL-4/IL-13 induced STAT6 signaling; and directly inhibit e-selectin, VCAM, and ICAM1 expression.[16-19] Additionally, corticosteroids upregulate the production of IL-10, as observed in our plasma proteomics, which reduces T_H2 cytokine production.[20] Interestingly, the anti-inflammatory effects of prednisolone in the airway varied among individuals despite confirmed prednisolone adherence in all participants. This means that these inflammatory pathways were active in some people only at stable state, suggesting heterogeneity among the severe eosinophilic asthma phenotype.

Prednisolone also suppressed mast cell activity. Prostaglandin synthases (PTGDS, HPGDS) and Siglec-6 were reduced, and 15-lipooxygenase was transcriptionally suppressed. Mast cell tryptase (CPA3) was not in the Olink panel, but the related CPB1, which resides at the same gene locus as CPA3,[21] was significantly reduced. Additionally, the beta tryptase gene in mast cells (*TPSB2*) was transcriptionally suppressed in nasal tissue (see Results section in the Online Repository). Corticosteroids reduce mast cell signaling pathways and attenuate histamine release.[22] *Post hoc* analysis of nasal transcriptome data in children suggested that mepolizumab may enhance mast cell and epithelial inflammation.[23] If corticosteroid-responsive mast cell activation remains present in some individuals despite mepolizumab therapy, this may be clinically relevant due to mast cell–mediated airway smooth muscle contraction and may drive some residual exacerbations that occur despite effective eosinophil depletion.

Proteomics showed that prednisolone reduced the matrix metalloproteinases MMP12 and MMP1 in sputum and plasma, which correlated with type 2 epithelial cytokines. Furthermore,

MMP12 transcription was significantly downregulated in nasal tissue after prednisolone (see Results section in the Online Repository). MMP12 is produced by macrophages and bronchial epithelial cells.[24] In murine models, it has been linked to airway remodelling via IL-13 dependent mechanisms.[25,26] In humans, reduced *MMP12* expression due to a functional polymorphism was associated with improved pulmonary function.[27] MMP1 expression is increased in the airways of people with asthma and is inversely associated with FEV_1.[28] *In vitro*, MMP1 is induced via β3-integrin–mediated MAPK signaling leading to reduced smooth muscle contraction.[29] Additionally, MMP1 is activated by mast cell tryptase, which generates pro-proliferative extracellular matrix (ECM).[30] Our data suggest that IL-13 and mast cell–dependent MMP12 and MMP1 activation remain in some patients with asthma despite anti-IL-5 treatment. The consequent potential effect on long-term lung function decline needs further investigation.

Prednisolone also significantly reduced GDNF and NCAM, mediators of neuroimmune activity, and these reductions correlated strongly with other type 2 inflammatory proteins. GDNF signals through the NCAM receptor.[31] *In vitro*, GDNF expression is increased in human airway smooth muscle cells in asthma and influences intracellular calcium responses.[32] In rat asthma models, GDNF expression was significantly higher than in controls, and central sensitization to GDNF injected in the lateral ventricle led to a significant increase in bronchial IL-4 and T_H2 cells and a significant decrease in pulmonary function.[33] GDNF may contribute to smooth muscle hyperreactivity, and we found that it was corticosteroid responsive.

Corticosteroids can inhibit signaling through the Toll-like receptor cascade, including tethering and inhibiting transcription factors such as NF-κB through glucocorticoid receptors.[34] Our data suggest that components of NF-κB signaling were downregulated by prednisolone, and, interestingly, this was the only protein associated with reduction in FENO. This association is plausible because inducible nitric oxide synthase (NOS2) production is dependent on NF-κB signaling.[35] The NOS2 protein was not in the Olink panel, so we cannot comment on its response to prednisolone. The mechanisms underpinning elevated FENO in some people despite anti-IL-5 treatment is of clinical interest. There was a suggestion in our proteomic and transcriptome pathway analysis that MAPK, which is important in Toll-like receptor signaling and transactivates STAT6,[36,37] and IL-4/IL-13 signaling pathways were upregulated following mepolizumab initiation (see Results section in the Online Repository). We also found upregulation of ciliary and ECM genes in the nasal transcriptome analysis that may represent epithelial repair mechanisms. This would be consistent with previous nasal transcriptome data in mepolizumab-treated children where increased expression of epithelial signaling and ECM pathways were associated with more exacerbations, likely due to increased cycles of epithelial repair after exacerbation.[38,39] In adults, nasal epithelial cytokine and alarmin activation (IL-4, TSLP, IL-33) after mepolizumab treatment was associated with nonresponder status,[40] suggesting a clinically relevant subgroup of severe eosinophilic asthma where inflammation is driven more by alarmins and the IL-4/IL-13 pathway than by IL-5–mediated eosinophilia.[41] As this epithelial signaling pathway was steroid responsive, we speculate that this inflammation could also be targeted using anti-IL4Rα, anti-TSLP, anti-IL-33, or Janus kinase inhibitors.

Our study has several strengths. First, the crossover study design allowed paired comparison of biological effects of prednisolone versus placebo within each participant, thus avoiding interparticipant variability. Second, high-throughput multiomic analyses of both airway and plasma samples enabled extensive exploration of prednisolone effects in both compartments. The only previous placebo-controlled proteomics study of prednisolone in asthma analyzed only plasma.[5] Third, objective measurement of plasma prednisolone levels in participants and the high intraclass correlation coefficients in most sputum and plasma Olink proteins provide confidence that effects are attributable to treatment.

The main limitation of our study was modest sample size. While the paired crossover design improved power for prednisolone versus placebo assessment, the lack of placebo control limited assessment of mepolizumab effects. Another potential limitation was that 4 participants were withdrawn after randomization. However, these withdrawals occurred before our bioinformatic analysis, so the risk of bias is less likely. Additionally, the non–ethnically diverse sample may limit the generalizability of the study. It is also possible that differences in demographics between patients providing sputum and plasma, such as age of asthma onset, could influence the differences between these compartments. Our findings are exploratory and should be replicated in future cohorts. A possible limitation is that mepolizumab may interfere with the Olink IL-5 assay. Additionally, a risk in any crossover study is the chance of period effects and carryover effects influencing results. However, we minimized period effects because the trial was performed in patients with stable asthma, treatment durations were short, and we used the treatment period as a fixed effect in our models.[42] Carryover effects were unlikely because we used a conservative 28-day washout period—far longer than the half-life and reported transcriptional effects of systemic steroids.[43,44] Finally, while sampling occurred within a clinical trial, allowing close comparison of biological and clinical effects, our study was undertaken at stable state without long-term follow-up during exacerbations, so we did not detect significant clinical changes.

The advent of anti-IL-5 mAb therapy has dramatically altered management of severe asthma. Patients rely less on OCS, with their extensive associated toxicity, to achieve asthma control. However, severe asthma is a heterogeneous condition. Our data demonstrate that some individuals have IL-5–independent airway epithelial inflammatory pathways that remain active and are responsive to corticosteroids. The limited effect of prednisolone on symptoms and lung function in MAPLE suggests that these residual corticosteroid-responsive pathways play a minor functional role in stable patients treated with mepolizumab. However, the effect of this persistent inflammation on key asthma outcomes such as long-term lung function decline and risk of exacerbations is unknown. Prospective studies are required to determine if there are clinically relevant anti-inflammatory effects of prednisolone at exacerbation in people treated with asthma mAbs.[45]

Data sharing

Raw data will be made available in the Gene Expression Omnibus on publication. Remaining data analyzed and presented in this study are available from the corresponding author on reasonable request, provided that the request meets local ethical

and research governance criteria after publication. Patient-level data will be anonymized.

DISCLOSURE STATEMENT

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Key messages

- **Prednisolone has extensive anti-inflammatory airway effects on top of mepolizumab.**
- **These effects are heterogeneous and could be clinically relevant in residual exacerbations.**

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