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ORIGINAL ARTICLE

Inhaled steroids associated with decreased macrophage markers in nonasthmatic individuals with sickle cell disease in a randomized trial

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Abstract

Inhaled mometasone was shown to improve pain scores and decrease soluble vascular cell adhesion molecule (sVCAM) concentration in a randomized controlled trial of nonasthmatic patients with sickle cell disease. We sought to explore potential changes in systemic inflammation as a mechanism underlying this effect. Serum samples from 41 trial participants (15 placebo- and 26 mometasone-treated) were analyzed using a 92 inflammatory marker panel at baseline and after 8 weeks of mometasone therapy. Individual marker analysis and correlation analysis were conducted. Adjusted for age, the mometasone-treated group decreased the concentration of CXCL9, CXCL11, CD40, IL-10, and IL-18 relative to placebo-treated participants. Hierarchical clustering and correlation analysis identified additional evidence for a decrease in cytokines linking to macrophage signaling and migration. There was no statistically significant change in markers of asthma and allergy, indicating that the improvement was unlikely mediated by modulation of occult reactive airway disease. This analysis of inflammatory markers suggests that decrease in macrophage activity may be involved in the mediation of the clinical benefit seen with use of inhaled mometasone in nonasthmatic patients with sickle cell disease.

Trial registration: clinicaltrials.gov identifier: NCT02061202.

Keywords Sickle cell disease · Macrophage activation · Hemoglobinopathies · Inhaled corticosteroids

Introduction

Sickle cell disease (SCD) is an autosomal recessive genetic disorder that causes ongoing hemolysis and vaso-oclussive crises (VOC) resulting in organ damage, pain, and decreased life expectancy. [1, 2]

Both murine models and human studies demonstrate that inflammation and increases in proinflammatory cytokine expression are fundamental components of SCD physiology; [3–8] however, the relative contributions of different components of the immune system are insufficiently characterized. One well-established mechanism is increased level of soluble vascular cell adhesion molecule (sVCAM) produced by the vascular endothelium, which facilitates hypoxia-induced adhesion to the endothelial surface, especially during VOCs. [9–12] Another common finding is increase in myeloid lineage cytokines, especially those indicative of increased macrophage activity such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β), [7, 11, 13–15] which may be driven partly by macrophage clearance of free heme groups that result from hemolysis. [16–18] Furthermore, activation of macrophages by clearing heme groups in has been shown to promote a proinflammatory macrophage phenotype in tissue culture and mouse models of SCD. [16]. Evidence of chronically activated circulating monocytes in SCD further supports the importance of the monocytic lineage in the pathophysiology of this disease. [19]

However, recent examination of lung tissue indicates that excess of CD4+ T cells, CD8+ T cells, and Treg may play a central role in mediating hyperresponsiveness and a

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proinflammatory state of the lung in SCD. [5] There is also evidence of dysregulation of CD8+ and Treg activity systemically in SCD, though aberrant ratios and subpopulations rather than increased absolute numbers are found. [20–23] Given these contrasting findings, it remains unclear whether the primary determinant of the inflammatory state in sickle disease is driven by myeloid or lymphoid elements.

As the only site in which sickling is reversed, the lungs represent not only a common site of disease involvement, but also a site where intervention could potentially be impacting widespread disease activity including both local and systemic inflammation. Distinct macrophage subsets are detectable at different stages of lung injury, inflammation, and recovery, as well as in patients with chronic lung diseases. [24–26] Notably, pulmonary macrophages are derived from and rapidly repopulated by blood monocytes, so changes in the pulmonary macrophage population may reflect a more systemic effect rather than solely a local change. [27]

Pulmonary inflammation and airway hyperreactivity and resistance appear to be consequences of SCD and, more importantly, mediators of morbidity and mortality in SCD. [3, 28–31] These airway changes are observed even in individuals without asthma. [32, 33] Furthermore, even in individuals without asthma, as well as those with it, there is evidence of respiratory symptoms or infections triggering other SCD complications, including VOC, acute chest syndrome, and death, for as much as months after symptoms. [34–39] As such, targeting pulmonary inflammation, even in SCD patients without asthma, may reduce VOCs and disease severity.

We previously reported a reduction in pain and sVCAM levels in a randomized trial of inhaled mometasone in patients without reactive airway disease. [40] Here, we present the effect of this intervention on a diverse set of inflammatory markers.

Methods

Intervention

Nonasthmatic patients with SCD who reported cough or wheeze within the prior 2 months were randomized to once daily mometasone furoate 220 mcg power inhalation or placebo. Patients, providers, and investigators were blinded to study assignment. Full protocol detail and adherence were previously described. [40] This trial was registered with clinicaltrials.gov (NCT02061202) and performed under an FDA investigational new drug application (IND 117997). This trial was approved by our institutional review board.

Patients

This analysis included only patients who were adherent to at least 70% of prescribed doses of study medication and for whom serum samples from both before and after 8 weeks of treatment were available for analysis (see Fig. 1). The study population did not include any patients with a known diagnosis of pulmonary hypertension, though this was not a prespecified exclusion criterion.

Measurement of inflammatory markers

Serum samples were analyzed for a panel of 92 circulating inflammatory proteins using the Olink multiplex assay (Olink Bioscience, Uppsala, Sweden).

An incubation master mix containing pairs of oligonucleotide-labeled antibodies to each protein was added to the samples and incubated for 16 h at 4 °C. Each protein is targeted with two different epitope-specific antibodies, which increased specificity. Presence of the target protein in the sample would bring the partner probes in close proximity, allowing the formation of a double strand oligonucleotide polymerase chain reaction (PCR) target. After incubation, the extension master mix in the sample would initiate the specific target sequences to be detected and generates amplicons using PCR in a 96-well plate. For the detection of the specific protein, a dynamic array integrated fluidic circuit (IFC) chip is primed, loaded with 92 protein-specific primers, and mixed with sample amplicons including three inter-plate controls (IPC) and three negative controls. Real-time microfluidic quantitative PCR (qPCR) is performed in Biomark (Fluidigm, San Francisco, CA) for the target protein quantification.

Data are analyzed using a real-time PCR analysis software via the $\Delta\Delta$Ct method and Normalized Protein Expression (NPX) manager. Assay pass and fail criteria were determined using four different internal assay reaction controls in every sample. Data is normalized using triplicate of IPC and triplicate of negative controls were used to determine the Limit of Detection (LOD). Ct values were converted to NPX and expressed as Log_2 scale which is proportional to the protein concentration. One NPX difference equals to the doubling of the protein concentration.

Statistical analysis

For each marker, we calculated the treatment effect as a difference in differences: the percentage change after the intervention in the control group was subtracted from the treatment group for each marker level. This approach was selected as it represents the deviation from baseline compared to placebo.

Despite randomization, uneven allocation of patient by age was noted in our study population, as previously published (see Table 1). [40] As such, percentage change in cytokine for

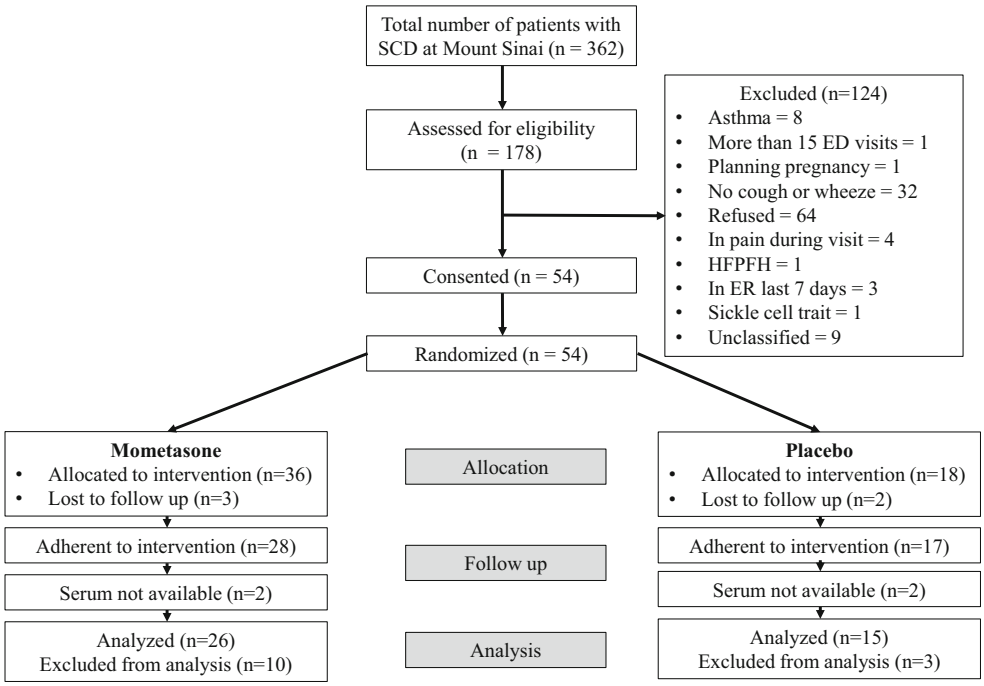

Fig. 1 Patient Flow CONSORT Diagram. Among those individuals randomized, lack of adherence to therapy excluded eight patients from the mometasone group and one from the placebo group. Two additional individuals in each group were not included in analysis due to lack of serum sample availability

placebo and mometasone groups were compared with multiple linear regressions adjusted for age. Adjustment for multiple hypothesis testing was not done. Regression modeling was conducted using Stata 11.0 (StataCorp, College Station, TX).

To assess whether broad expression profiles changed with intervention, inflammatory serum markers were also analyzed with complete-linkage hierarchical clustering based on spearman correlation distance. Spearman correlation analysis of inflammatory markers and hierarchical clustering was conducted using R version 3.4.0.

Results

Demographic characteristics of participants have been previously reported (see Table 1). Randomization was successful with the exception of age, as already noted.

Individual marker analysis

Of the 92 analytes assessed, we report all those found to be statistically significant and other pertinent negatives. The remaining analytes are reported in the supplemental appendix.

In individual marker analysis of chemokine ligands, CXCL9 and CXCL11 were noted to be statistically significantly decreased with mometasone in comparison with placebo (see Table 2). After adjusting for age, the treatment group had an average decrease in CXCL9 and CXCL11 of 12.2 and 8.0% more than placebo, respectively. Other chemokine ligands did not demonstrate a statistically significant treatment effect.

Among interleukins, IL-10 and IL-18 demonstrated a significant reduction. Additionally, CD40 was the only member of the tumor necrosis factor (TNF) superfamily to show a statistically significant decrease attributable to the intervention.

Table 1 Summary statistics

Characteristic	Placebo (n = 17)	Mometasone (n = 35)	p
Male sex, n (%)	11 (64%)	17 (49%)	0.4
Age, mean (SD)	36 (9.81)	30 (8.56)	0.02
Hydroxyurea use, n (%)	11 (64.7%)	23 (65.7%)	1.0
History of allergies, n (%)	6 (35%)	14 (40%)	0.3
Smoking, n (%)			0.8
Never	9 (52%)	22 (63%)	
Former	6 (35%)	10 (29%)	
Current	2 (12%)	3 (9%)	
Hemoglobin at entry (g/dL), mean (SD)	8.92 (1.59)	8.55 (1.54)	0.4
sVCAM at entry (mg/mL), mean (SD)	2378.9 (987.4)	2712.7 (1478.8)	0.4

Please note that full summary statistics were previously published in Glassberg et al. 2017

Table 2 Analysis of chemokines/cytokines

Chemokine ligands		
	Treatment effect (%)	*p*
CCL20	− 4.5	0.251
CCL28	7.5	0.251
CXCL9	− 12.2	0.024*
CXCL11	− 8.0	0.024*
MCP-1	− 1.1	0.559
MCP-3	16.3	0.25
MCP-4	− 4.2	0.337
Cytokines—TNF superfamily		
	Treatment effect (%)	*p*
CD40	− 1.9	0.029*
TNF	0.2	0.238
TNF-β	− 1.5	0.517
TNFRSF9	− 0.8	0.782
TNFSF14	5.1	0.4
Cytokines—interleukins		
	Treatment effect (%)	*p*
IL-2	14.4	0.159
IL-2Rβ	19.0	0.172
IL-4	0.3	0.916
IL-5	2.4	0.531
IL-6	3.9	0.696
IL-7	− 3.5	0.603
IL-10	− 20.0	0.021*
IL-10RA	24.7	0.274
IL-12β	− 1.3	0.635
IL-13	0.6	0.133
IL-17A	32.8	0.136
IL-18	− 4.3	0.012*
IL-18R1	− 3.4	0.072
Cytokines—other		
	Treatment effect (%)	*p*
IFN-γ	2.4	0.172
TSLP	28.1	0.16

*$p < 0.05$

Of note, interferon gamma (IFN-γ) was not significantly changed during the study period; this finding corroborates our previous findings using an ELISA assay. [40]

We analyzed CCL19, CCL25, CDCP1, CCL11, FGF21, FGF23, Flt3L, IL-10Rβ, and IL-6 as these markers were recently shown to distinguish between well- and poorly controlled asthma. [41] Among these markers (see Table 3), none were significantly changed between the treatment and placebo arms. IL-6 also plays a role in iron recycling during erythrophagocytosis in macrophages. Specifically, IL-6 expression in Raw264.7 cells, a macrophage cell line, was recently shown to increase in response to heme. [16] This prior finding, that IL-6 production by macrophages is enhanced during high heme states, as occurs during hemolysis in SCD, suggests that mometasone treatment does not alter heme processing and iron recycling in macrophages. These results confirm previously demonstrated IL-6 results in these study subjects. [40]

Results of additional marker analysis are available in the supplemental appendix.

Correlation analysis

While multiple regressions allowed for the assessment of change in individual analyte levels with respect to treatment while correcting for age, we also wanted to establish whether certain analytes, while perhaps not significantly changed between treatment groups, change together, indicating connections across analytes and possibly better elaborating potential mechanisms.

Hierarchical clustering of patients based on correlation distances of all percent changes between pre- and post-treatment values was performed, and inspection of the resulting heatmap of analyte changes showed incomplete segregation of treatment and placebo patients (see Fig. 2). Though not uniformly present, this represents a change in the broad inflammatory signature of mometasone-treated patients. Consistent with the individual analyte analyses, CXCL9, CXCL11, CD40, IL10, and IL18 are reduced in the subset of mometasone-treated patients who were most strongly segregated from patients in the placebo arm. This indicates that the effect observed in the individual analysis is driven by the same set of patients with a change in a broader profile of markers. Furthermore, when correlation between analytes was assessed (see Fig. 3), the majority of these analytes (CXCL9, CD40, IL10, IL18) were encompassed in a large module of analytes that were correlated. As such, while only a small number of individual markers were statistically significantly changed, this suggests a broader shift in the expression of myeloid lineage cytokines.

Table 3 Asthma control markers

	Treatment effect (%)	*p*
CCL11	− 0.9	0.544
CCL19	− 0.9	0.629
CCL25	− 4.3	0.099
CDCP1	4.0	0.560
FGF-21	− 11.0	0.484
FGF-23	10.0	0.477
Flt3L	− 0.9	0.522
IL-6	3.9	0.696
IL-10Rβ	− 1.4	0.263

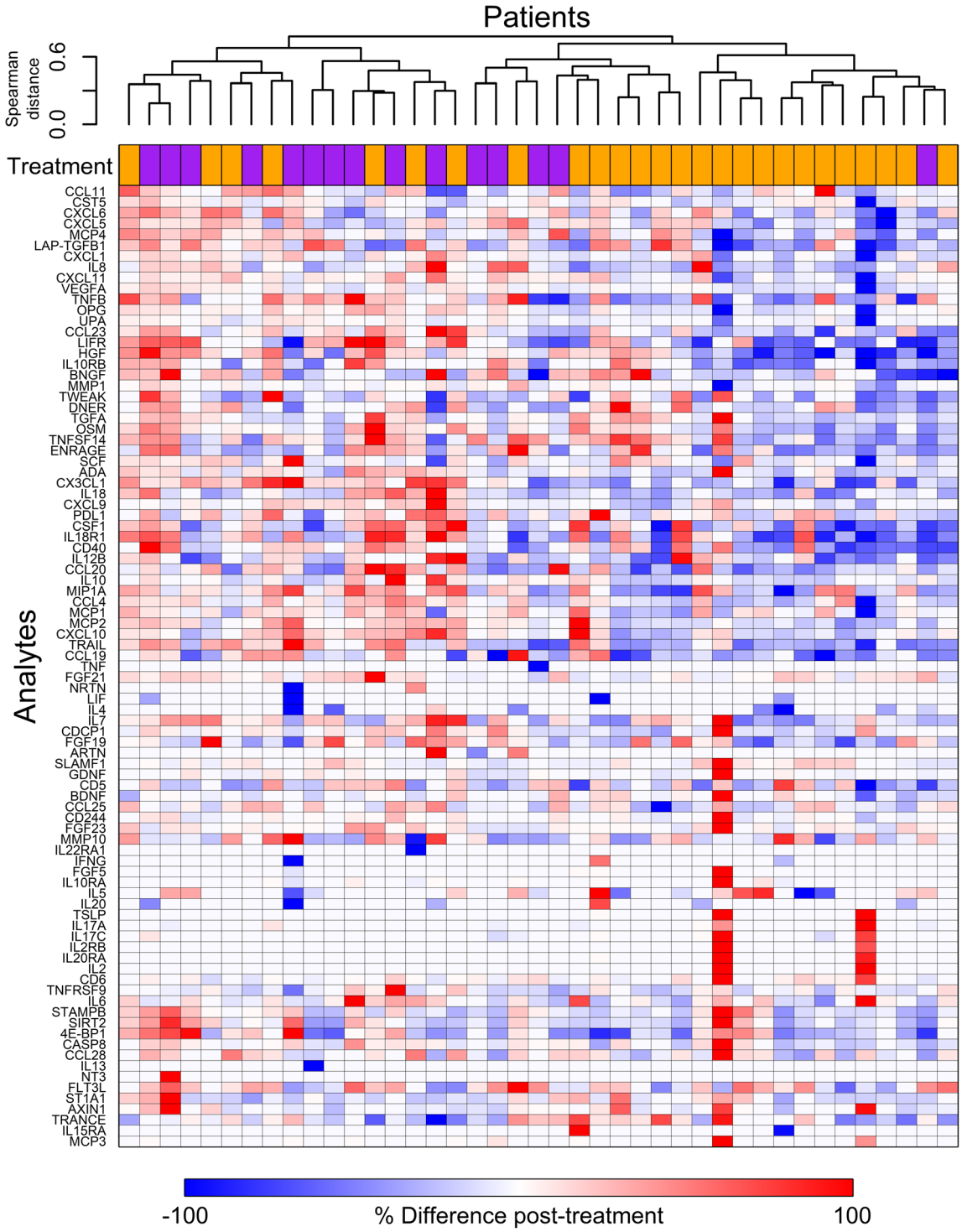

Fig. 2 Hierarchical clustering of patients by post-treatment analyte signature demonstrates grouping based on treatment. Heatmap of percent-differences in Olink analyte measurements post-treatment with either inhaled mometasone (orange) or placebo (purple). Patients and analytes are ordered by complete-linkage hierarchical clustering according to Spearman correlation distance. Based on the clustering of increases (red) and decreases (blue) in analytes, the majority of patients treated with mometasone are able to be segregated from those who received placebo. This is primary driven by markers associated with mononuclear phagocytes

Discussion

With this analysis, we sought to elucidate potential mechanisms through which low-dose inhaled steroids lead to systemic benefits. Treatment with inhaled mometasone was associated with a broad change in the inflammatory profile expressed. Both individual analytes (i.e., CXCL9, CXCL11, CD40, IL10, and

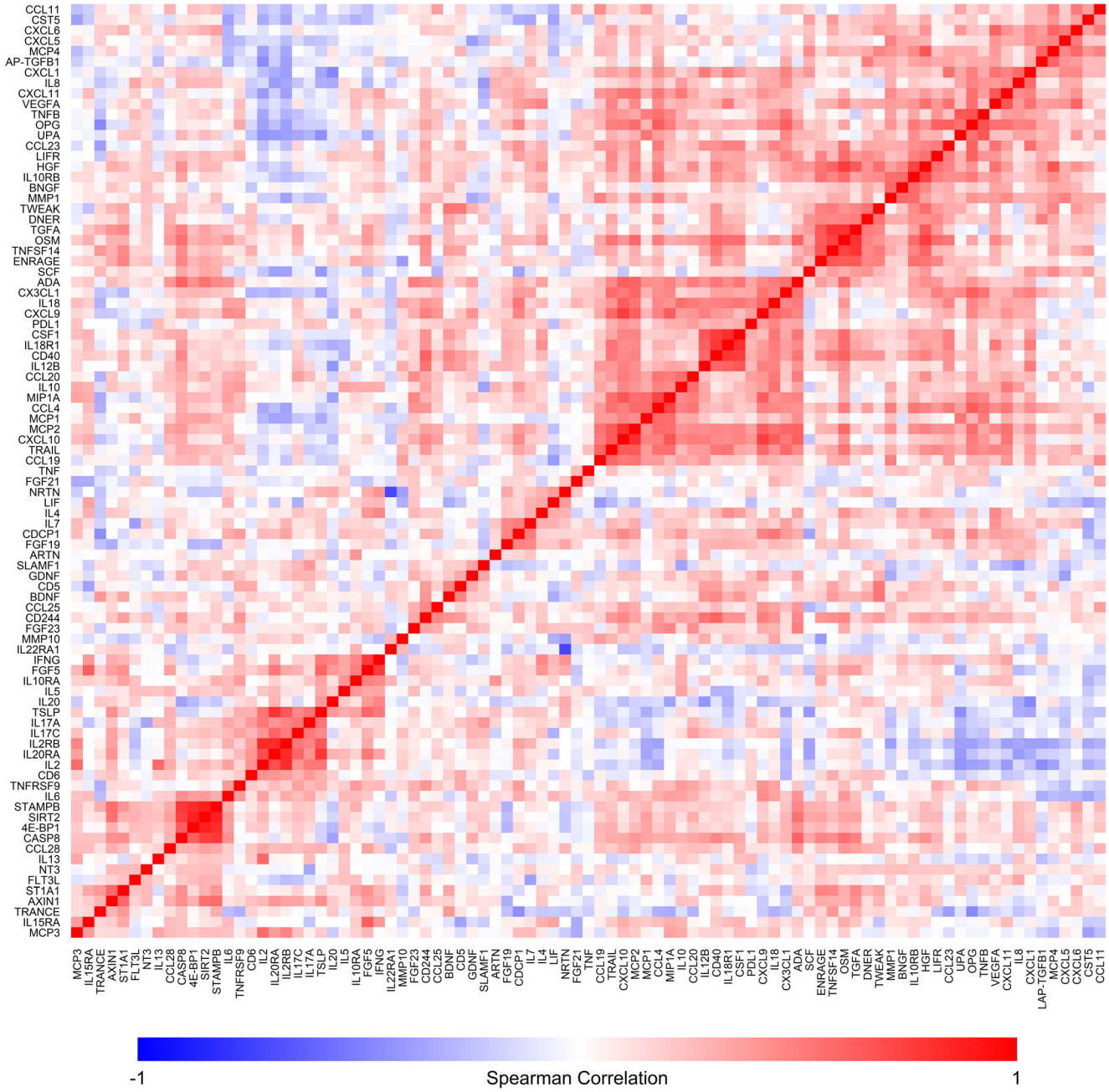

Fig. 3 Correlation analysis reveals disease-associated analyte modules. Heatmap of Spearman correlation values of post-treatment percent-differences in analyte measurements. Analytes are ordered by complete-linkage hierarchical clustering

IL18) and clusters of correlated analytes found to be significantly decreased in the treatment population suggest that the change observed affects mononuclear phagocytes (MNP; monocytes, macrophages, and dendritic cells) in which these molecules are co-expressed upon exposure to inflammatory stimuli. Although additional analytes were not significantly changed between treatment groups, the correlation of these molecules with other MNP-implicating molecules such as MCP1 and CCL20 (see Fig. 3) that are produced by macrophages further supports an MNP-centered mechanism. As such, we suspect the effect of mometasone involves altered macrophage function or responsiveness as evidenced by changes in cytokines secreted by macrophages.

IL-6 levels were not statistically different between groups but IL-6 was numerically higher in the steroid group. That IL-6 did not decrease with inhaled

corticosteroids is consistent with data demonstrating that IL-6 mediates macrophage scavenging of free heme and would increase when hemolysis is reduced. [16] Alternatively, the absence of significant alteration in IL-6 may indicate that the impact on the MNP axis was mediated locally, rather than systemically. As our samples were all derived from peripheral blood, we are unable to assess whether this reflects changes in lung macrophages, blood monocytes or both. This would be an area meriting future exploration, given evidence of the distinct role of pulmonary macrophages. [24, 25]

The reduction in IL-10 levels must be considered further, as IL-10 is considered an anti-inflammatory cytokine, but has demonstrated a distinct profile in SCD. Expression of IL-10 is increased at baseline in patients with SCD compared with controls, [42] despite the clear chronic proinflammatory profile of this disorder. An evaluation of IL-10 during VOCs found a decrease from baseline, [43] while levels were increased in patients receiving hydroxyurea. [44] These contrasting findings emphasize the importance of the MNP axis, but suggest greater complexity than IL-10 simply being a marker of disease activity. It is possible that these alternations in IL-10 levels may not play a causal role nor indicate downstream effects of the causal pathway in the benefits of hydroxyurea or mometasone.

Notably, we confirmed the lack of change in IFN-γ originally assayed by ELISA in our initial study. [40] While IFN-γ is expressed in many contexts including during macrophage activation, the present data suggests that it is not part of the causal pathway in this clinical trial due to its relative lack of correlation with the above myeloid group. Alternatively, the small sample size of this study may have resulted in insufficient power to detect a difference.

We do not observe significant changes in the T cell axis, including but not limited to Tregs. However, some of the research indicating alterations in T cell profiles was conducted with samples of lung tissue, a local microenvironment, while our specimens were from peripheral blood. [5] While further mechanistic and tissue studies are needed to fortify this conclusion, we hypothesize that the reported T cell accumulation in SCD tissues is due to nonspecific inflammation resulting in a general recruitment of T cells as opposed to clonal proliferation of T cells at the tissue site.

By design, patients with asthma or a reactive airway disease were excluded from the study population, as the goal of the intervention was to identify whether patient without another indication for inhaled corticosteroids could benefit from this therapy. As such, the absence of statistically significant changes in markers associated with asthma control [41] and allergic spectrum disorders is consistent with successful exclusion of those with reactive airway disease and that the effect of the intervention was not mediated by treating uncontrolled reactive airway disease. Notably, while this analysis can rule out with some confidence subclinical reactive airway disease, it does not identify which cells mometasone was directly acting on to yield the improvement in pain scores seen in the trial.

There are several limitations of this analysis. The inflammatory panel analyzed here contains 92 different markers. Thus, there is a risk of introducing type II error. Of note, the observation that the effects observed were all decreases provides evidence that the findings are not all due to random chance, as this would be expected to occur in both directions. We did not correct for multiple hypothesis testing, as we consider this analysis to be exploratory and hypothesis generating that would require corroboration in a larger trial regardless of the threshold used for statistical significance. Additional trial would also be of use to address the small sample size of this study and provide additional time points for serum sampling.

We must also note that the study population was restricted to individuals over age 15, so we cannot infer whether these findings apply to the pediatric population.

Another limitation is that the decreases observed here do not prove causality or mediation. The decreases in CXCL9, CXCL11, CD40, IL-10, and IL-18 as well as the broader change in the expression profile demonstrated by correlation analysis may be incidental to the mechanism of reducing pain and sVCAM or caused-by instead of causing the changes. While our study only looked at analytes detected in peripheral blood, we cannot make an inference based on our data with respect to whether the effect of the treatment is mediated by a local effect in the lung and reduced hemoglobin polymerization. An intrapulmonary effect could still be related to immune modulation or lead more directly to improved oxygen exchange and, therefore, decreased sickling.

In conclusion, we find that inhaled mometasone in nonasthmatic patients with SCD is associated with a decrease in macrophage markers but no change in asthma control markers. This suggests that occult reactive airway disease is not mediating the clinical benefits of this intervention previously reported. While prospective analysis in future trials of inhaled corticosteroids will be needed to confirm the validity of these findings, our results provide evidence for the involvement of the macrophage axis in the clinical benefit seen with this intervention.

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Compliance with ethical standards

Conflict of interest The authors declare they have no conflicts of interest.

Ethical approval All procedures performed in this study were in accordance with the ethical standards of the institutional review board and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individuals included in the study.

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