Suboptimal treatment response to anti-IL-5 monoclonal antibodies in severe eosinophilic asthmatics with airway autoimmune phenomena

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Severe asthmatics with adult-onset asthma, sinus disease and requiring daily prednisone, are at higher risk for responding suboptimally to current doses of anti-IL-5 mAbs, with further risk of worsening on an IgG_1 mAb if they have sputum autoantibodies https://bit.ly/2Ahpvsm

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ABSTRACT

Background: In clinical trials, the two anti-interleukin (IL)-5 monoclonal antibodies (mAbs: mepolizumab and reslizumab) approved to treat severe eosinophilic asthma reduce exacerbations by ∼50–60%.

Objective: To observe response to anti-IL-5 mAbs in a real-life clinical setting, and to evaluate predictors of suboptimal response.

Methods: In four Canadian academic centres, predefined clinical end-points in 250 carefully characterised moderate-to-severe asthmatic patients were collected prospectively to assess response to the two anti-IL-5 mAbs. Suboptimal response was determined based on failure to reduce maintenance corticosteroid (MCS) or asthma symptoms scores (Asthma Control Questionnaire (ACQ)) or exacerbations, in addition to persistence of sputum/blood eosinophils. Worsening in suboptimal responders was assessed based on reduced lung function by 25% or increase in MCS/ACQ. A representative subset of 39 patients was evaluated for inflammatory mediators, autoantibodies and complement activation in sputum (by ELISA) and for immune-complex deposition by immunostaining formalin-fixed paraffin-embedded sputum plugs.

Results: Suboptimal responses were observed in 42.8% (107 out of 250) patients treated with either mepolizumab or reslizumab. Daily prednisone requirement, sinus disease and late-onset asthma diagnoses were the strongest predictors of suboptimal response. Asthma worsened in 13.6% (34 out of 250) of these patients. The majority (79%) of them were prednisone-dependent. Presence of sputum anti-eosinophil peroxidase immunoglobulin (Ig) G was a predictor of suboptimal response to an anti-IL-5 mAb. An increase in sputum C3c (marker of complement activation) and deposition of C1q-bound/IL-5-bound IgG were observed in the sputa of those patients who worsened on therapy, suggesting an underlying autoimmune-mediated pathology.

Conclusion: A significant number of patients who meet currently approved indications for anti-IL5 mAbs show suboptimal response to them in real-life clinical practice, particularly if they are on high doses of prednisone. Monitoring blood eosinophil count is not helpful to identify these patients. The concern of worsening of symptoms associated with immune-complex mediated complement activation in a small proportion of these patients highlights the relevance of recognising airway autoimmune phenomena and this requires further evaluation.

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Introduction

Targeting the interleukin (IL)-5 signalling pathway is now an established therapeutic strategy for patients with severe asthma whose severity is predominantly driven by eosinophils. Mepolizumab and reslizumab are neutralising monoclonal antibodies (mAbs) directed against IL-5, while benralizumab is an afucosylated mAb directed against the IL-5 receptor [1]. All three molecules effectively deplete blood eosinophil levels and, on average, reduce exacerbations by ∼50–60% [2–4]. There could be a number of reasons why the response is not more impressive. These include 1) selection of patients based on peripheral blood eosinophil counts which may not always be associated with airway eosinophilia [5, 6]; 2) inadequate dosing that may not suppress airway eosinophils [7]; 3) IL-5 not being the dominant cytokine driving eosinophilia [8]; and 4) airway autoimmune mechanisms (reported in the airways of up to one-third of patients with eosinophilic asthma [9]) that may interfere with the effects of the biologics [7, 10]. Local autoimmune response may interfere when IL-5 in the airway (which is a predictor of response to anti-IL-5 therapies [7]) is not adequately neutralised by anti-IL-5 mAb, and instead, when in a zone of equivalence with the antigen [11] may form IL-5–anti-IL-5 heterocomplexes with endogenous immunoglobulin (Ig)G autoantibodies and consequently activate the complement pathways [12]. This could lead to not just a suboptimal response, but potentially worsening of asthma. These phenomena would not be observed with benralizumab (since it is not directed against the antigen, IL-5, rather against the receptor), and therefore this article does not report clinical responses to benralizumab.

The primary objective of our study was to examine the prevalence and clinical predictors of suboptimal response and worsening of asthma in a real-world setting in 250 patients with moderate-to-severe asthma from four Canadian university hospitals who were prescribed mepolizumab or reslizumab based on best clinical practice and national regulatory guidelines. The secondary objective was to examine the molecular predictors, including autoimmune responses, in those patients who were suboptimal responders. In a smaller, but representative, subset of patients with available sputum samples, we assessed inflammatory mediators (cytokines and released eosinophil products). We also analysed autoantibodies, immune-complex formation/deposition and complement activation. Finally, this study was not designed to test the superiority of one mAb over another, but only to report the rate of response to anti-IL-5 mAbs based on patient-related outcomes routinely used by clinicians in a pragmatic real-world scenario. Some of the results of this analysis have been reported previously in the form of an abstract [13].

Methods

Study patients and collection of clinical data

Either mepolizumab or reslizumab was prescribed as add-on therapy in patients with severe asthma (adult) with significant eosinophilia (blood and/or sputum), inadequately controlled despite treatment with a high-dose inhaled corticosteroid (ICS) plus another controller (equivalent to the Global Initiative for Asthma criteria 4 and 5) [14], according to the Health Canada prescription guidelines. Mepolizumab was available in Canada for patients as early as January 2016, whereas reslizumab became available ∼12 months later. All the initial prescriptions were for mepolizumab. When both drugs were available, prescriptions of the anti-IL-5 therapy was at the physician's discretion; dependent on the patient's insurance coverage and logistical considerations of administering subcutaneous *versus* intravenous dosing. Forced expiratory volume in 1 s (FEV_1) and Asthma Control Questionnaire (ACQ)-5 [15] were obtained as a part of routine follow-up in the clinical centres. In 160 subjects, sputum was induced and processed [16] as a part of routine clinical assessment. Fractional exhaled nitric oxide (F_{ENO}) was not routinely collected as part of clinical assessment, except in patients who could not produce sputum. These data are not included in this article. The clinical data were collected prospectively as and when the patients were routinely followed in their respective clinics, from the time when they were started on the adjunct anti-IL-5 therapy (pre-treatment value or baseline) until the database was locked or they were taken off the treatment due to inadequate/suboptimal response (between November 2015 and January 2019) (post-treatment values).

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For 250 patients who were included in the final analysis (figure 1) any additional data (refer to table 1 for list of clinical parameters) were collected as a part of retrospective chart review with ethical permission from the respective local ethics board. Matched pre- and post-treatment sputum for 39 patients (with

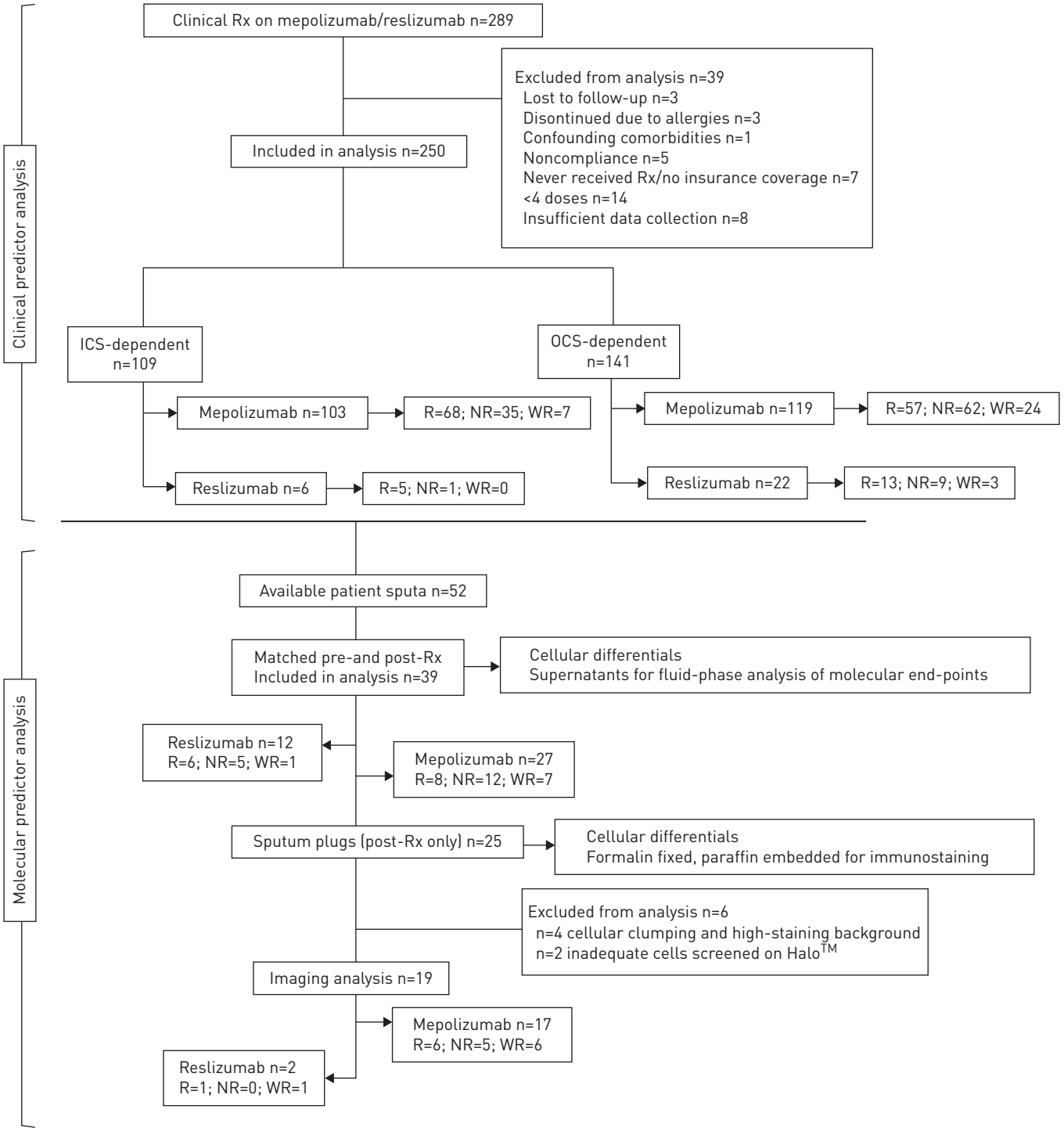

FIGURE 1 CONSORT diagram: patient recruitment for analysis of clinical and molecular predictors are given. 289 patients were recruited from four academic centres in Canada: McGill University Health Centre, Montreal, QC; Firestone Institute for Respiratory Health, Hamilton, ON; Sacré-Coeur Hospital of Montreal, Montreal; and Centre de Recherche de l'Institut Universitaire de Cardiologie et de Pneumologie de Québec, Laval, QC. Rx: treatment; ICS: inhaled corticosteroid; OCS: oral corticosteroid; R: responder; NR: nonresponder or suboptimal response; WR: worsened (in the clinical predictor analysis the total number of NRs includes those who worsened). Some patients were excluded for more than one reason.

TABLE 1 Patient demographics stratified as per response to anti-interleukin (IL)-5 monoclonal antibody (mAb) treatment

	Responders		Nonresponders		p-value[¶]
	Observed sample size[#]	Statistic	Observed sample size	Statistic	
Subjects n	143		107		
Male	143	77 (54)	107	54 (50)	0.688
Age years	143	57±13.9	107	54±12.6	0.119
BMI kg·m^{-2}	134	29.0±6.1	104	29.1±6.1	0.840
Oral corticosteroids (prednisone)	143	71 (50)	107	70 (65)	0.018*
Dose of prednisone mg·day^{-1}	71	11.3±6.8	70	13.8±10.1	0.087
Duration of prednisone years	45	6.55±5.3	55	8.01±7.1	0.245
Asthma onset in adulthood	112	90 (80)	90	80 (89)	0.145
Inhaled corticosteroid[+] µg·day^{-1}	143	1500 (1000–3000)	107	1500 (1000–3000)	0.653
Baseline FEV_1 % predicted	141	66.8±18.8	106	64.9±20.0	0.452
Previous biologic use	141	54 (38)	106	52 (49)	0.118
Lymphopenia	137	70 (51)	106	63 (59)	0.244
Atopy[§]	103	55 (53)	84	55 (65)	0.128
Presence of sinus disease[f]	141	65(46)	107	66 (62)	0.021*
Pre blood eosinophils ×10^9·L^{-1}	140	0.56±0.5	106	0.57±0.7	0.909
Pre sputum eosinophils % of total cell count	91	21.7±22.7	69	18.7±19.5	0.376
Exacerbations in preceding year	143	2.154±1.9	107	2.4±2	0.324
Exacerbations in preceding year	143	2 (0–12)	107	2 (0–9)	0.354

Data are presented as n, n (% of observed sample size), mean±SD or median (range), unless otherwise stated. Out of the 250 patients, 55 were recruited from the McGill University Health Centre, 88 from the Firestone Institute for Respiratory Health, St. Joseph's Healthcare (McMaster University), 47 from Sacré-Coeur hospital of Montreal and 60 from Centre de Recherche de l'Institut Universitaire de Cardiologie et de Pneumologie de Québec (Université Laval) were included in the final analysis. BMI: body mass index; FEV_1: forced expiratory volume in 1 s; Pre: pretreatment baseline values. [#]: the data available for each variable across sites; [¶]: two-sided t-test for continuous variables, Chi-squared test for dichotomous variables; [+]: presented as fluticasone propionate-equivalent; [§]: assessed as positive skin-prick test to common aeroallergens, or specific immunoglobulin E positivity in the sera; [f]: defined as physician-reported or computed tomography evidence of chronic sinusitis with or without polyps. *: indicates significant difference p<0.05.

adequate volume) for molecular analysis was available at the McMaster site (figure 1) and all experimental procedures were reviewed and approved by the Hamilton integrated research ethics board.

Steroid reduction strategy

After three doses of the anti-IL-5 mAb (*i.e.* 3 months), the maintenance dose of steroid was reduced by 5 mg every month, and asthma control was assessed with 3-month follow-up visits. For patients on long-term (>2 years) high-dose maintenance prednisone (>15–20 mg), tapering was halted at 5 mg·day^{-1}, and then patients were tested for adrenal insufficiency before completely weaning them off. For patients maintained on high-dose ICS (⩾1500 µg fluticasone propionate equivalent), the tapering was done at 500 µg (reduction by 1 puff twice daily every clinic visit). Asthma control during steroid tapering was monitored every 3 months using ACQ, sputum and/or spirometry.

Assessment of response to anti-IL-5 mAb therapy

Suboptimal response (NR) to therapy was determined by one of the three clinical criteria (failure to reduce maintenance corticosteroid by 50%, failure to reduce ACQ-5 ⩽1.5, failure to reduce exacerbations by 50%) plus persistence of sputum eosinophils >3% or blood eosinophils ⩾400 cells·µL^{-1} after ⩾4 months of treatment (adapted from a previous study [7]). Without a biological criterion (reduction in eosinophilia), the presence of any two of the three clinical criteria were accepted as designating NR status. Worsening on treatment was defined as patients identified as NR having a further one of the following criteria: reduction in FEV_1 from pre-treatment baseline by ⩾25%; any increase in maintenance corticosteroid; and increase in ACQ by 0.5 (minimal clinically important difference).

Assessment of inflammatory markers

Type 2 T-helper cell cytokines and mediators were evaluated using the Discovery assay (Eve Technologies, Calgary, AB, Canada), as described previously [9, 17], while eosinophil peroxidase (EPX) as a measure of airway eosinophil activity was assessed using ELISA [18, 19]. Anti-EPX IgG [9] and Ig-bound C1q (using

ELISA, #ab170246; Abcam, Cambridge, MA, USA) were detected in the sputa along with C3c, a marker of complement activation (#HK368-01; HyCult Biotech Inc., Wayne, PA, USA. Detailed methodology for detection and quantification of immune-complexes in the paraffin-embedded formalin-fixed sputum plugs is given in the supplementary material.

Statistical analysis

Detailed information on statistical analysis and strategies are provided in the supplementary material for both clinical and experimental cohorts reported here. Briefly, we calculated descriptive statistics of demographic and baseline clinical characteristics within groups of responders and suboptimal responders to anti-IL-5 therapy. Means±SD were used to summarise continuous variables, while frequencies and percentages were used to summarise categorical variables. From the logistic regression models, we reported the odds ratio and 95% confidence intervals associated with each clinical predictor (detailed method in the supplementary material). For the molecular end-points, the Kruskal–Wallis test with Dunn's multiple comparison was used between responders, suboptimal or nonresponders (NR) and those who worsened (WR). Prism (version 8; GraphPad, La Jolla, CA, USA) was used for statistical analysis and generation of plots.

Results

Study patients (recruitment and inclusion into analysis)

Based on the recent consensus statement by Buhl *et al.* [20] that severe eosinophilic asthmatics prescribed biologics should receive treatment for ⩾4 months, in the principal analysis we included 250 patients (out of 289) with at least four injections of either reslizumab/mepolizumab. The baseline demographic data for these 250 patients (figure 1) stratified by their response to anti-IL-5 mAb therapies is provided in table 1. In addition, the baseline data distribution between the four academic sites and baseline demographics for 148 patients who had ⩾12 injections are provided in supplementary tables E1 and E2, respectively.

Patient response to anti-IL-5 mAb treatment: suboptimal response

43% of patients showed suboptimal response to anti-IL-5 mAbs (table 1). Furthermore, for 148 patients who received ⩾12 months of therapy, the rate of suboptimal response, 42.5% (n=63), was comparable (Chi-squared, 0.002, degrees of freedom (df) 1; p=0.96). The clinical variables determined to designate clinical response are stratified based on the treatment groups in table 2 (subgroup analysis: supplementary table E4).

In ICS-dependent asthma: frequency of suboptimal response

The proportion of patients with suboptimal response was 33% (n=36) in 109 patients maintained on daily high-dose ICS (median dose 1500 µg of fluticasone equivalent), with 20 patients failing to reduce exacerbations by 50% and 12 failing to reduce at all (or documented an increase; table 2).

In oral corticosteroid-dependent asthma: frequency of suboptimal response

Out of 141 oral corticosteroid (OCS)-dependent asthmatics (median daily dose 10 mg), 50.3% showed inadequate response to either of the prescribed anti-IL-5 mAbs, with 52% (62 out of 119) nonresponse to mepolizumab and 41% (nine out of 22) to reslizumab (figure 1). Out of the 141 OCS-dependent patients who showed a suboptimal response to anti-IL-5 therapy, ∼34 (24%) could not reduce their OCS dose at all, and 56 (40%) failed to reduce it by 50% (table 2). The rate of NRs in the OCS group was higher than those maintained on ICS only (Chi-squared 7.4, df 1, z=2.6; p=0.008). The mean reduction of OCS in the responders was 74.3% compared to 12.2% in the suboptimal group (p<0.0001).

Patient response to anti-IL-5 mAb treatment: worsening of symptoms

34 (13.6%) patients worsened while on prescribed anti-IL-5 mAbs (table 2), of whom 27 (79%) were maintained on daily prednisone. Based on the three criteria for designating worsening, 1) 12 (35.2%) showed an increase in ACQ (median increase by 0.8 points); 2) 14 (41.1%) had their maintenance corticosteroid dose increased (median increase in prednisone by 7.5 mg, ICS by 500 µg·day^{-1}; 3) 11 (32.3%) recorded a fall in FEV_1 by ⩾25% (median fall of 31.2%, range 25–59%). The mean FEV_1 post-treatment recorded for 11 patients was 50.1±18% pred (Δ877±385 mL). Finally, only three patients were designated as "worsened" based on more than one of the three clinical criteria.

Clinical predictors for suboptimal response to anti-IL-5 treatment

Use of daily prednisone (OR 1.92, 95% CI 1.15–3.22), dose of prednisone (OR 1.04, 95% CI 1–1.08) and presence of sinus disease (OR 1.88, 95% CI 1.13–3.14) could independently, in a univariate analysis predict "suboptimal" response to an anti-IL-5 mAb (table 3). This is further reflected in the baseline difference (table 1) for OCS (p=0.02) and sinus disease (p=0.02) between the two response groups (table 1). A multivariate regression model (table 3) showed that there was an increased possibility of a

TABLE 2 Distribution of clinical variables determining nonresponse to biologics

	Suboptimal response to anti-IL-5 mAb		Suboptimal response in ICS-dependent patients		Suboptimal response in OCS-dependent patients	
	Observed sample size	Statistic	Observed sample size	Statistic	Observed sample size	Statistic
Subjects	107 (42.8)[#]		36 (14.4)[#]		71 (28.4)[#]	
Length of treatment months	107	14 (4–59)	36	12 (4–55)	71	15 (4–58)
Worsening on treatment	250	34 (13.6)	36	7 (19.4)	71	27 (38.0)
Stratifying contributing factors for "suboptimal" response: clinical criteria						
Failure to reduce MCS <50%	107	90 (84.1)	36	34 (94.4)	71	56 (78.8)
MCS dose reduction =0%, or increase	107	63 (58.8)	36	29 (80.5)	71	34 (47.8)
Failure to reduce ACQ below 1.5	72	61 (84.7)	20	19 (99.0)	52	42 (80.7)
Failure to reduce exacerbations by 50%	105	66 (62.8)	36	20 (55.5)	69	46 (66.6)
Failure to reduce exacerbations =0%, or any increase	105	49 (46.6)	36	12 (33.3)	69	37 (53.6)
Increase in exacerbations	105	28 (26.6)	36	6 (16.6)	69	22 (31.8)
Stratifying contributing factors based on biological criteria (suboptimal response)						
Sputum eosinophils ⩾3%	67	51 (76.1)	18	12 (66.6)	49	39 (79.6)
Blood eosinophils ⩾0.4	99	8 (8.0)	32	4 (12.5)	67	4 (5.9)
Criteria for worsening in addition to suboptimal response						
Increase in ACQ >0.5	23	12 (52.2)	7	2 (28.5)	16	10 (62.5)
Increase in MCS	34	14 (41.1)	7	5 (71.5)	27	9 (33.3)
Reduction in FEV_1 ⩾25%	34	11 (32.3)	7	0	27	11 (40.7)
Length of treatment months	34	11 (4–26)	7	10 (4–22)	27	23 (4–30)

Data are presented as n (% of observed sample size), n or median (range). Observed sample size is based on data available/collected per variable. Suboptimal response population includes the worsening subpopulation. IL: interleukin; mAb: monoclonal antibody; ICS: inhaled corticosteroids; OCS: oral corticosteroids; MCS: maintenance corticosteroids; ACQ: Asthma Control Questionnaire; FEV_1: forced expiratory volume in 1 s. [#]: percentage based on total number of patients assessed for response.

suboptimal response in late-onset asthmatics (OR 4.5, 95% CI 1.14–17.79) with evidence of sinus disease (OR 3.47, 95% CI 1.23–9.78) and/or atopy (OR 3.28, 95% CI 1.11–9.68). In the OCS-dependent group, subgroup analysis revealed ∼28-fold increase in risk of suboptimal response (OR 27.87, 95% CI 1.3–599.25) if the patient has adult-onset asthma, and ∼12-fold risk (OR 12.66, 95% CI 2.17–74.02) with evidence of sinus disease.

Molecular mechanisms underlying inadequate response to anti-IL-5 treatment

For molecular investigations, a smaller subset of prototype patients was recruited with clinical characteristics representative of the larger clinical cohort of 250 patients (supplementary table E3).

Assessing eosinophil-associated inflammatory mediators as fluid phase markers

An increase in sputum IL-5 was noted in the mepolizumab suboptimal responders post-treatment (figure 2) (p=0.04), indicating inadequate neutralisation of the antigen. Univariate analysis showed a significant predictive value only for granulocyte–macrophage colony-stimulating factor (GM-CSF) (table 4) (estimate±SE −6.3±2.9, p<0.03). However, the absolute values (figure 2d) are predominantly below the lower limit of detection, and hence the role of GM-CSF remains inconclusive. As depicted in figure 2g, sputum levels of EPX were unremarkable at baseline and were not computed to be a predictor of suboptimal response (table 4) (OR 0.97, 95% CI 0.77–1.22; p=0.8).

Assessing autoimmune-mediated inflammation (fluid phase)

Anti-EPX IgG was used as a marker of localised autoimmunity [7, 9] (figure 3a). Levels of anti-EPX IgG were elevated in the airways (p=0.03) of patients who failed to respond adequately to mepolizumab (figure 3). Additionally, in mepolizumab-treated patients who showed worsening of symptoms, both C1q-IgG and C3c levels were significantly elevated from baseline (figure 3).

TABLE 3 Clinical predictors for nonresponse to anti-interleukin (IL)-5 treatment

	Univariate analysis	Multivariate analysis[#]	Multivariate subgroup analysis (patients on OCS)[¶]	Multivariate subgroup analysis (patients with sputum analysis)
Subjects n	250	133	77	119
Clinical variables (pretreatment values)				
Male *versus* female	0.87 (0.53–1.44)	0.69 (0.25–1.89)	0.25 (0.05–1.16)	0.91 (0.37–2.22)
Age per year increase	0.99 (0.97–1.01)	0.97 (0.93–1.01)	0.91 (0.84–0.99)	0.98 (0.95–1.02)
BMI per unit increase	1 (0.96–1.04)	0.89 (0.81–0.98)	0.89 (0.79–1)	0.88 (0.81–0.96)
Smoking status				
Never-smoker *versus* ex-smoker	1.33 (0.79–2.25)	3.03 (0.98–9.36)	1.38 (0.21–8.91)	2.56 (0.92–7.16)
Smoker *versus* ex-smoker	1.39 (0.47–4.14)	4.45 (0.41–48.49)	2.23 (0.09–53.33)	4.21 (0.44–0.33)
Corticosteroid use				
OCS *versus* ICS	**1.92 (1.15–3.22)**	2.61 (0.86–7.93)	NA	**2.99 (1.08–8.31)**
Prednisone dose	**1.04 (1–1.08)**	NA	1.06 (0.96–1.18)	1.06 (0.95–1.11)
Duration of prednisone	1.04 (0.97–1.11)	NA	NA	1.1 (0.96–1.26)
Additional clinical parameters				
Onset of asthma, adulthood *versus* childhood	1.96 (0.88–4.39)	**4.5 (1.14–17.79)**	**27.87 (1.3–599.25)**	2.98 (0.88–10.13)
Pre-blood eosinophils per unit increase	1.03 (0.68–1.56)	1.27 (0.47–3.44)	1.0 (0.34–2.97)	1.0 (0.43–2.33)
Previous biologic use	1.55 (0.93–2.58)	1.5 (0.56–4.05)	0.73 (0.17–3.09)	1.01 (0.41–2.52)
Lymphopenia	1.4 (0.84–2.34)	0.79 (0.28–2.19)	0.16 (0.03–0.93)	0.61 (0.24–1.56)
Atopy	1.66 (0.92–3.01)	**3.28 (1.11–9.68)**	3.22 (0.76–13.67)	**3.38 (1.24–9.21)**
Presence of sinus disease	**1.88 (1.13–3.14)**	**3.47 (1.23–9.78)**	**12.66 (2.17–74.02)**	**3.2 (1.2–8.51)**
Baseline FEV_1 % pred per unit increase	0.99 (0.98–1)	0.97 (0.94–1)	0.97 (0.93–1.01)	0.97 (0.95–0.99)
Pre-sputum eosinophils per unit increase	1.96 (0.88–4.39)	NA	NA	0.99 (0.97–1.01)

Data are presented as n or OR (95% CI). Bold font indicates clinical variable that may predict inadequate response to a prescribed anti-IL-5 treatment. OCS: oral corticosteroids; BMI: body mass index; ICS: inhaled corticosteroids; FEV_1: forced expiratory volume in 1 s. [#]: sputum data were not collected at McGill University Health Centre, Montreal (MUHC), while the Laval site did not collect prednisone duration data. To include these sites in the multivariate analysis, we did not add "pre-sputum eosinophils" or "prednisone duration" as independent variables in the second column. The multivariate analysis was done in those patients with complete datasets available for all remaining 14 variables; [¶]: to allow MUHC site patient dataset into the regression analysis, this set of analyses did not include sputum eosinophils as an independent variable.

Commensurate with the distribution plots for all inflammatory markers assessed (figures 2 and 3), a multivariate regression analysis confirmed baseline anti-EPX IgG levels (at 1:2 titre) to be a predictor for suboptimal response to an anti-IL-5 mAb (estimate±SE −1.77±0.7, z-value −2.2; p=0.02) (table 4 and supplementary table S3).

Assessing in situ *immune-complex deposition in fixed sputum plugs*

Figure 4 details the co-localisation of C1q-IgG and IL-5 IgG in the airways of patients who suboptimally responded to mepolizumab; this was not done in reslizumab since there was no evidence of C1q-IgG or C3c in their sputa (figure 3). The absolute values of the differential cell count for the respective sputum samples (from which the plug was selected for embedding) have been given. As is evident, there is no particular increase in a cell-type which limits us to the assessment of whether there is a particular cell associated with the C1q-IgG/IL-5 IgG co-localisation. A significant correlation (r=0.8, p<0.0001) between the $IL\text{-}5^{+}IgG^{+}/C1q^{+}IgG^{+}$ dual-positive cells indicates a mutually inclusive event (figure 4c, representative image in supplementary figure S3).

Discussion

We report suboptimal response in a population of moderate-to-severe eosinophilic asthmatics to adjunct anti-IL-5 mAb treatment (107 (42.8%) out of 250). In addition, we report a novel observation of worsening of asthma in 13.6% (34 out of 250) of patients with "eosinophilic asthma" on anti-IL-5 neutralising mAbs. Oral corticosteroid dependence, late onset of asthma, and sinus disease (paradoxically,

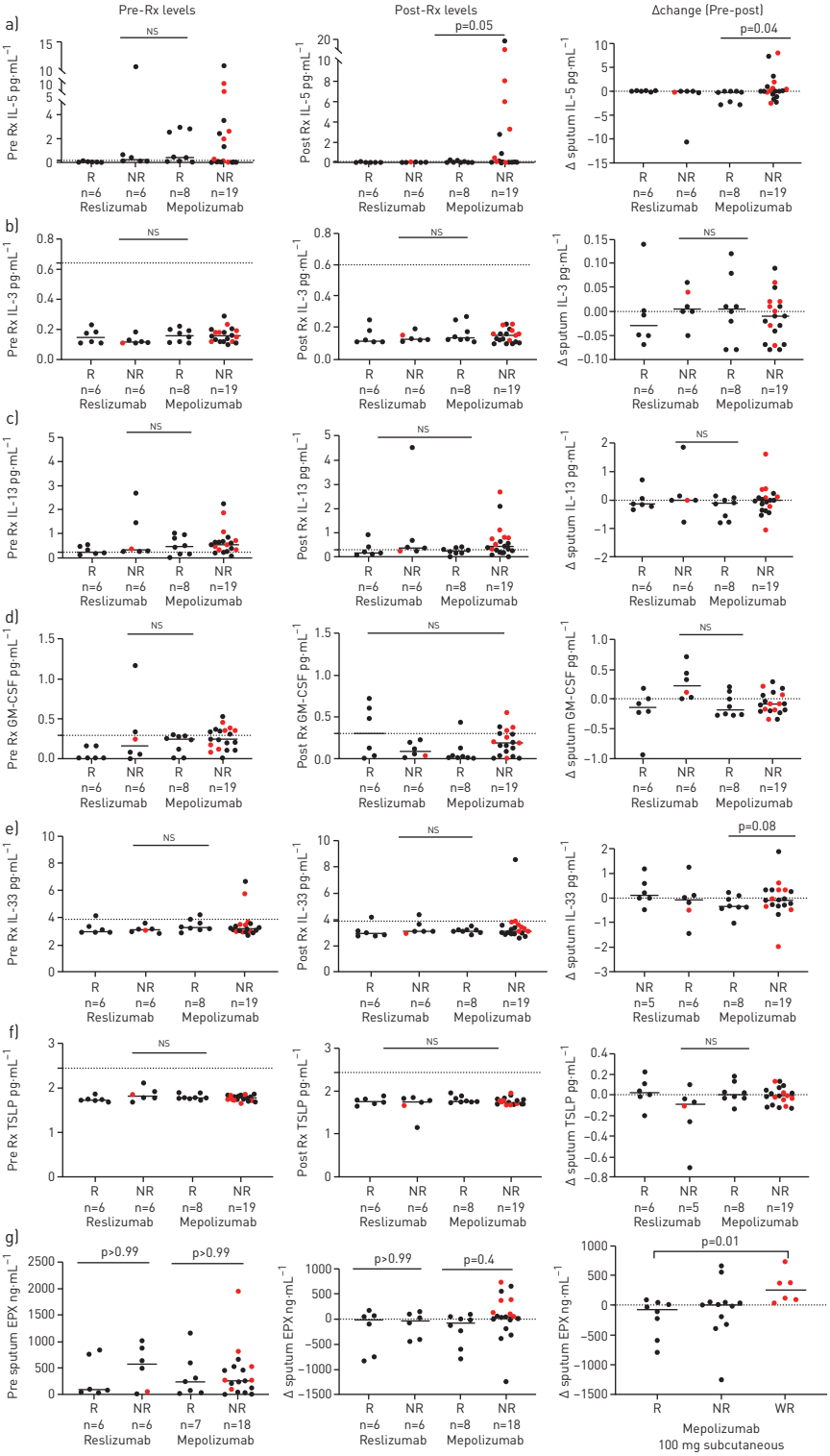

FIGURE 2 Airway assessment of eosinophilopoietic cytokines and mediators pre- and post-treatment with anti-interleukin (IL)-5 monoclonal antibodies (mAbs). The pre-treatment, post-treatment and the change (Δ) post-treatment from baseline are plotted for a) IL-5, b) IL-3, c) IL-13, d) granulocyte–macrophage colony-stimulating factor (GM-CSF), e) IL-33, f) thymic stromal lymphopoietin (TSLP) detected using Discovery multiplex assay (Eve Technologies, Calgary, AB, Canada), done in duplicate. Values are stratified based on response (R) and suboptimal (non)response (NR). Dotted lines indicate individual lower limit of detection on the validated luminex platform for pretreatment plots. g) Sputum eosinophil peroxidase (EPX) levels, marker of ongoing airway eosinophil activity, are plotted using similar stratification strategy. Red symbols indicate individual patients who worsened on the drug as per the clinical criteria. Kruskal–Wallis with Dunn's multiple correction. $p<0.05$ is considered significant. Rx: treatment.

TABLE 4 Molecular predictors for nonresponse to anti-interleukin (IL)-5 treatment

	Univariate analysis		Multivariate analysis#	
	OR (95% CI)	p-value	OR (95% CI)	p-value
Subjects n	39		39	
Variables at baseline (pretreatment)				
Markers for autoimmune responses (sputum)				
Anti-EPX IgG	0.14 (0.02–0.56)	0.0122¶	0.18 (0.03–0.69)	0.0254¶
Anti-MARCO IgG	0.80 (0.26–2.17)	0.670		
C1q-Ig	0.89 (0.76–1.00)	0.0919		
C3c	1.01 (0.99–1.04)	0.2454		
Eosinophilopoietic cytokines and mediators (sputum)				
EPX	0.97 (0.77–1.22)	0.764		
IL-3	5.92 (8.88×10^{-7}–2.39×10^{7})	0.817		
IL-4	0.72 (0.15–1.42)	0.520		
IL-5	0.70 (0.34–1.02)	0.184		
IL-9	2.61 (0.08–113.27)	0.550		
IL-13	0.27 (0.03–1.20)	0.167		
GM-CSF	0.002 (3.25×10^{-6}–0.29)	0.0286¶	0.002 (1.89×10^{-6}–0.71)	0.0610
IL-33	0.99 (0.33–2.39)	0.98		
TSLP	0.06 (3.15×10^{-6}–239.35)	0.540		
TARC	0.99 (0.93–1.04)	0.8158		

EPX: eosinophil peroxidase; Ig: immunoglobulin; MARCO: macrophage receptor with collagenous structure; IL: interleukin; GM-CSF: granulocyte–macrophage colony-stimulating factor; TSLP: thymic stromal lymphopoietin; TARC: thymus and activation-regulated chemokine. #: multivariate analysis with all variables with p<0.05 as per individual univariate analysis of independent variables. For further details on the model, intercept, standard error and β-coefficients, refer to supplementary table S3; ¶: significant variables that may predict inadequate response to anti-IL-5 treatment.

these are the best indicators of an IL-5-driven eosinophilia) were the strongest predictors of suboptimal response. Presence of autoimmune responses in the airways, formation of heterocomplexes and complement activation contributed to the worsening of asthma.

The response rate to anti-IL-5 mAb therapies reported in this study is consistent with phase III clinical trials [2–4] and real-life cohort studies [21–25]. However, the criteria used in our study and the other observational studies were different, and therefore it is challenging to contrast our data with the other publications. Unlike our study, the other observational studies do not provide any insight into mechanisms of poor response. Our investigations reveal that suboptimal responders to both anti-IL-5 therapies had increased baseline anti-EPX IgG levels. Furthermore, anti-EPX IgG was observed to be the only molecular factor that could predict a possible nonresponse to anti-IL-5 mAbs (table 4). A significant positive correlation between EPX levels and anti-EPX IgG (r=0.45, p=0.02) suggested an ongoing autoimmune response that sustains the ongoing eosinophilic activity, and the events remain uncurbed despite the high-dose corticosteroid therapy and additional anti-IL-5 mAb. The clinical indicators for nonresponse to anti-IL-5 therapy (*e.g.* OCS use and sinus disease) further reflects a population that is prone to localised autoimmune inflammation as reported in recent studies [9, 26]. This phenomenon is localised to the airways as there was no increase noted in the systemic markers of autoimmune responses (anti-neutrophil cytoplasmic antibodies, rheumatoid factors or complement activation). C-reactive protein, a marker of acute inflammation, was unremarkable between the pre-treatment and post-treatment levels (pre-Rx median (interquartile range (IQR)) 2.3 (5.5); post-Rx median (IQR) 3.9 (8.9); p=0.36).

These autoantibodies, being of the IgG subtype, can bind to complement (C1q). In the event that the drug is inadequate for the target antigen [11], there may be immune-complexes formed with the IgG_1 mAb as well as the IgG autoantibodies, forming heteroimmune-complexes (for example, IL-5–IgG/EPX–anti-EPX IgG), bound to the six heads of a C1q molecule [27]. C1q activation can either induce the complement cascade or heighten inflammation by recruiting immune cells *via* FcRγ receptors without binding other complement factors [28]. Increased anti-EPX IgG, C3c and C1-q/IgG levels in the fluid phase, along with C1q–IgG/IL-5–IgG dual-positive cells (in the sputum plugs, see supplementary figure E4 for representative pre- *versus* post-mepolizumab image), strongly suggest an autoimmune-triggered immune-complex

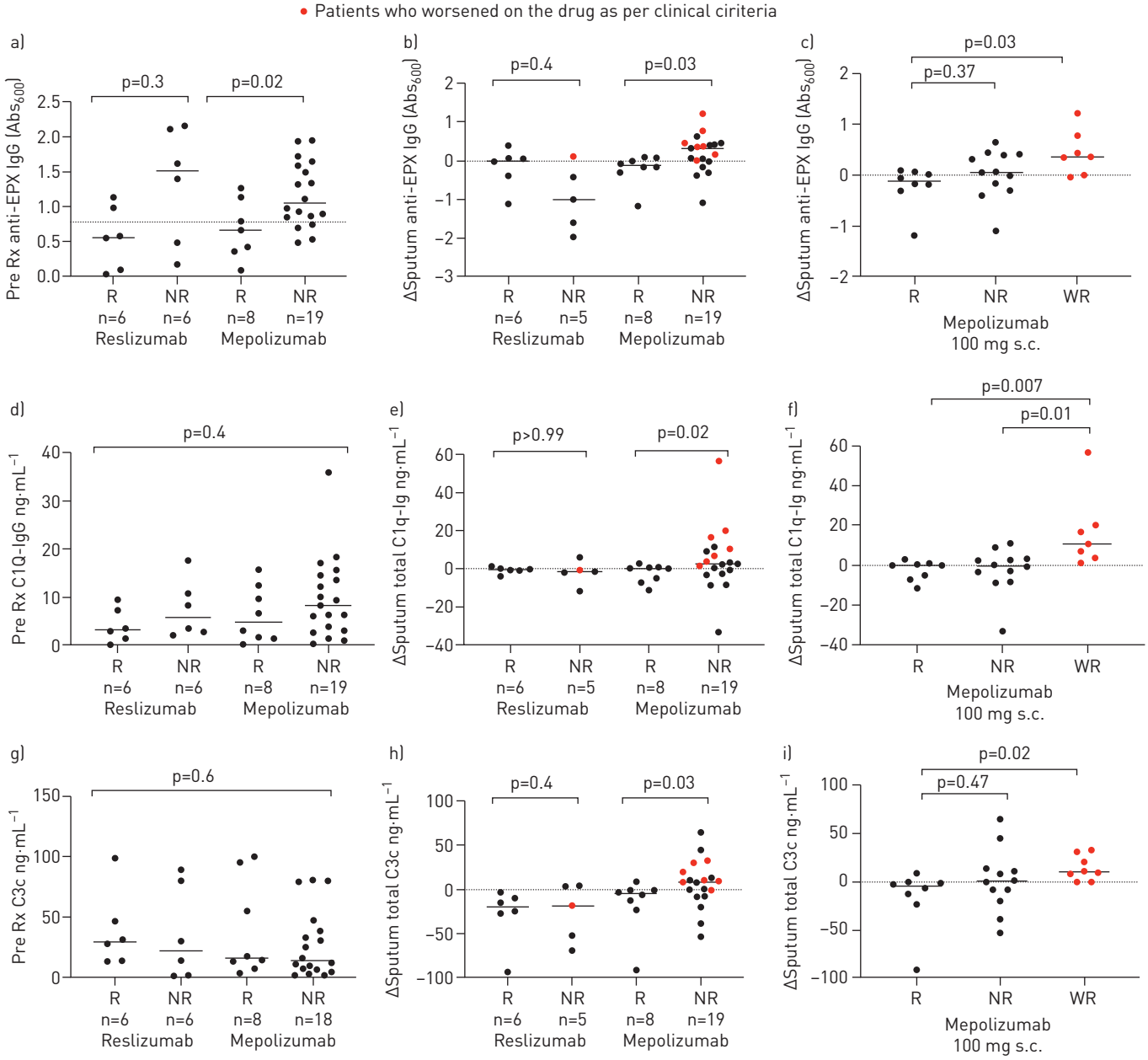

FIGURE 3 Markers of autoimmune responses pre- and post-treatment with anti-interleukin (IL)-5 monoclonal antibodies (mAbs). Sputum anti-eosinophil peroxidase (EPX) immunoglobulin (Ig)G levels are plotted for patients with adequate sample volume available pre- and post-treatment with mepolizumab/reslizumab. Anti-EPX IgG absolute values (1:2 titre, cut-off threshold indicated by dotted line) in the sputum a) prior to anti-IL-5 therapy is plotted stratified on treatment type and the individual clinical response; differences in absolute values obtained from deducting post-treatment values from pre-treatment values are plotted for b) both treatment types and c) those treated with mepolizumab only. Values are stratified based on response (R), suboptimal or nonresponse (NR) and worsening (WR) on the respective treatment. Immune-complex mediated increase in inflammatory status was assessed by detecting airway levels of d,e,f) C1q bound to Ig fraction of sputum and g,h,i) free C3c in sputum supernatants (marker of complement activation) and are plotted using similar stratification strategy. Red symbols indicate individual patients who worsened on the drug as per the clinical criteria. Reslizumab cohort was not plotted separately due to low n values. Kruskal–Wallis with Dunn's multiple correction. $p<0.05$ is considered significant. The post-values reported for NRs (reslizumab) is n=5, as compared to the pre-treatment group (n=6), due to low sample volume from one patient which was inadequate for the end-point assessment. All plotted data points are mean of n=2 duplicate values. Rx: treatment; s.c.: subcutaneous.

mediated pathology in the patients who worsened on mepolizumab. Worsening on reslizumab could not be conclusively linked to a similar pathology due to limited sample size. The three reported cases of worsening (representative image of low/no immune-complex deposition is given in supplementary figure E5) is unlikely to be mediated by immune-complex mediated complement activation, possibly because reslizumab has an IgG_4 backbone which does not bind (C1q) complement [29].

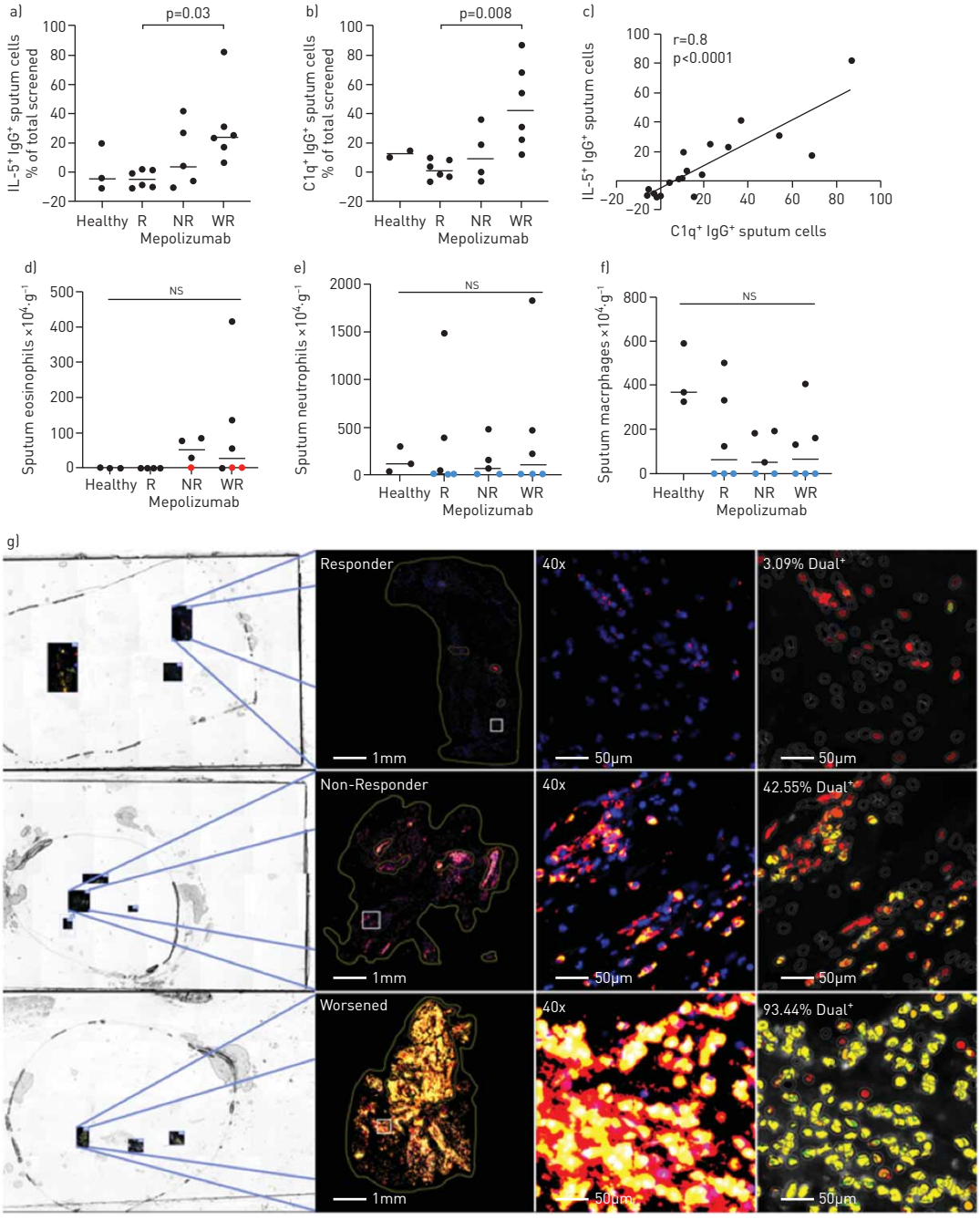

FIGURE 4 Immune-complex mediated worsening of patients post-mepolizumab therapy. Immune-complex deposition in the airways assessed by immunostaining formalin-fixed, paraffin-embedded (FFPE) sputum plug sections with 1) rabbit anti-human C1q antibody (secondary antibody: anti-rabbit AlexaFluor 488, visualised in the green channel) and mouse anti-human immunoglobulin (Ig)G (anti-mouse AlexaFluor 546, red channel), and 2) rabbit anti-human IL-5 antibody (anti-rabbit AlexaFluor 488, green) and anti-human IgG (red). Co-localisation (yellow) of C1q (green) and IgG (red), and IL-5 (green) and IgG (red) on matched patient (sputum plug FFPE) slides were scanned and assessed using cellular analysis on the HALO platform. Values are represented as a percentage of total number of cells screened. As a background control, the percentage of dual-positive cells in the secondary control slide (no primary antibody added) was subtracted from the total number counted in the stained slides. Yellow hot-spots or dual-positive cells indicating co-localisation are plotted for a) IL-5$^+$ IgG$^+$ cells and b) C1q$^+$ IgG$^+$ cells. Values are stratified based on the treatment response to mepolizumab (response (R), suboptimal or nonresponse (NR) and worsening (WR)). c) Correlation between IL-5$^+$ IgG$^+$ and the C1q$^+$ IgG$^+$ cells (*i.e.* indicative of immune-complexes). For all the FFPE sputum plugs, remaining sputum was processed for a differential count and are plotted: d) absolute sputum eosinophil counts, where red symbols indicate sputum with high salivary contamination that were not deemed fit for sputum differential count, but showed many free eosinophil granules, indicative of continual eosinophil activity; e) absolute sputum neutrophil counts; f) absolute sputum macrophage counts, where blue symbols indicate sputum with high salivary contamination that were not deemed fit for sputum differential count, and therefore the values on the graph are nonrepresentative of the definite airway numbers. g) Representative micrographs of patient samples based on response to mepolizumab is given for C1q-IgG staining. DAPI (blue) is used for staining the nuclei. Please refer to supplementary figure E1 for reference to visualisation and method validation.

In addition, we tested the hypothesis that poor response might have been due to IL-5 not being the dominant driver of eosinophilia in these patients. Evidence from sputum transcriptomics data from the U-BioPred study [30] strongly suggest that in addition to IL-5, other cytokines related to innate lymphoid cell group 2 biology such as IL-33, thymic stromal lymphopoietin and IL-13 may be mediators of/contributors to eosinophilia. However, except for GM-CSF, none of the other cytokines related to eosinophil biology were predictors of suboptimal response. We believe that IL-5 is indeed the dominant cytokine in the severe eosinophilic patients as we observed an increase in sputum IL-5 in the patients who showed inadequate responses to mepolizumab (figure 2a), indicative of poor neutralisation of target antigen, and perpetuation of ongoing eosinophilic inflammation. Finally, in patients who worsened (indicated by red symbols in figure 2c) there was an increase in IL-13 in the sputa, which agrees with the cytokine signature we previously reported for asthmatics with sputum autoantibodies [9].

Finally, absolute blood eosinophil counts, currently recommended to be the best biomarker for anti-IL-5 mAb treatment response, is not supported by our current observation in a dataset of 250 patients. Indeed, post-treatment blood eosinophils were elevated ($\geqslant$400 cells·μL^{-1}) in only 8% of the suboptimal responders (table 2). In contrast, 76% of the 67 suboptimal responders with available airway inflammometry data (table 2), showed increased sputum eosinophils $\geqslant$3%, indicating unsuppressed airway eosinophilia. Furthermore, 68.6% of these patients had sputum eosinophils despite normalisation of blood eosinophils. These further agree with previous reports on discordance between blood and sputum eosinophils [5], and the former to be an inadequate biomarker for monitoring therapeutic response to anti-IL-5 mAbs [7, 31, 32], particularly in prednisone-dependent asthmatics, as depicted in table 2. In similar prospective real-life studies, baseline blood eosinophil levels were not predictive of response to mepolizumab [21, 22, 33]. Although F_{ENO} correlates with airway eosinophilia in steroid-naïve patients, it is not helpful to monitor response to therapies with anti-IL5 mAb [34] and therefore, it was not routinely measured in this study.

Although this study is one of the largest detailed description of clinical responses to anti-IL-5 mAbs, we acknowledge the limitation due to incomplete data collection of a few variables in approximately a third of patients, particularly sputum cytology, as this is not part of routine clinical assessment in all academic centres. We have attempted to address this in our statistical analysis outlined in the supplementary material. A second limitation is the low numbers of patients who were on reslizumab, and therefore the current study does not attempt to make any direct comparisons between the clinical efficacies of the two mAb therapies. However, even with the limited samples, we were able to establish that the underlying factors leading to inadequate response and/or worsening for these two anti-IL-5 mAbs appear to be different. Finally, although we speculate that underdosing may be responsible for the suboptimal response to the anti-IL-5 mAbs, we have not provided pharmacokinetic data to support this hypothesis. Validated assays of mAb concentrations in airway secretions are not commercially available and we were not able to obtain these assays from the manufacturers of the said therapeutic molecules.

In summary, we report a significant prevalence of suboptimal response to the two currently approved anti-IL-5 neutralising mAbs in moderate-to-severe asthmatics associated with blood and/or sputum eosinophilia. This is unlikely to be due to IL-5 not being the dominant cytokine in perpetuating eosinophilia. Indirect evidence suggests that inadequate neutralisation of IL-5 in the airways may be relevant. More importantly, we report the presence of an alternative inflammatory event, airway autoimmunity, that may compromise anti-IL-5 treatment efficacy, and in some leads to worsening of symptoms and airway obstruction *via* immune-complex mediated injury. Monitoring of blood eosinophil count may not be helpful to identify this. Clinicians ought to be mindful of this possibility while prescribing anti-IL-5 mAb therapy to asthmatics who are prednisone-dependent, with late onset asthma diagnosis, evidence of a sinus disease, and therefore likely to have a higher burden of IL-5 in their airways.

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Author contributions: P. Nair and M. Mukherjee developed the concept, M. Mukherjee designed all experiments. P. Nair, J.G. Martin, C. Lemiere and L-P. Boulet recruited patients. S. Tran, M-E. Boulay, M. Bertrand, H. Al-Hayyan, J. Cherukat, M. Kjarsgaard and C. Huang collected and tabulated the data. K. Radford and M. Mukherjee performed molecular experiments. T. Javkar performed all immunostaining protocols while K. Ask, S.D. Revill and A. Ayoub did the microscopy and related validation of image analysis. A. Dvorkin-Gheva, N. Dendukuri and D.F. Forero undertook all statistical analysis. M. Mukherjee wrote the first draft. P. Nair, J.G. Martin, K. Ask, C. Lemiere, L-P. Boulet and N. Dendukuri edited and added to the development of the manuscript. All authors have read and agreed to the submitted manuscript. P. Nair and M. Mukherjee take overall guarantee of the manuscript.

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