

Innate and adaptive T cells in asthmatic patients: Relationship to severity and disease mechanisms

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Background: Asthma is a chronic inflammatory disease involving diverse cells and mediators whose interconnectivity and relationships to asthma severity are unclear.

Objective: We performed a comprehensive assessment of T_H17 cells, regulatory T cells, mucosal-associated invariant T (MAIT) cells, other T-cell subsets, and granulocyte mediators in asthmatic patients.

Methods: Sixty patients with mild-to-severe asthma and 24 control subjects underwent detailed clinical assessment and provided induced sputum, endobronchial biopsy, bronchoalveolar lavage, and blood samples. Adaptive and invariant T-cell subsets, cytokines, mast cells, and basophil mediators were analyzed.

Results: Significant heterogeneity of T-cell phenotypes was observed, with levels of IL-13-secreting T cells and type 2 cytokines increased at some, but not all, asthma severities. T_H17 cells and $\gamma\delta$ -17 cells, proposed drivers of neutrophilic inflammation, were not strongly associated with asthma, even in

severe neutrophilic forms. MAIT cell frequencies were strikingly reduced in both blood and lung tissue in relation to corticosteroid therapy and vitamin D levels, especially in patients with severe asthma in whom bronchoalveolar lavage regulatory T-cell numbers were also reduced. Bayesian network analysis identified complex relationships between pathobiologic and clinical parameters. Topological data analysis identified 6 novel clusters that are associated with diverse underlying disease mechanisms, with increased mast cell mediator levels in patients with severe asthma both in its atopic (type 2 cytokine-high) and nonatopic forms.

Conclusion: The evidence for a role for T_H17 cells in patients with severe asthma is limited. Severe asthma is associated with a striking deficiency of MAIT cells and high mast cell mediator levels. This study provides proof of concept for disease mechanistic networks in asthmatic patients with clusters that could inform the development of new therapies. (*J Allergy Clin Immunol* 2015;■■■:■■■-■■■.)

Key words: Asthma, T lymphocytes, cytokines, mast cells, phenotype, endotype, regulatory T, T_H17, T_H2, mucosal-associated invariant T-cell

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Asthma is characterized by airways inflammation and remodeling. Based on initial studies in animal models¹ and human T-cell clones² and bronchoscopic studies in patients with mild steroid-naïve asthma,^{3,4} it has been viewed as a disease driven by activated T_H2 cells producing the type 2 interleukins IL-4, IL-5, and IL-13. These cytokines are believed to orchestrate the functions of mast cells,^{5,6} eosinophils,⁷ and IgE-producing B cells/plasma cells. This concept has been challenged with increasing recognition of considerable heterogeneity of asthma mechanisms and definable patient subpopulations associated with immunopathology that cannot be explained by T_H2 inflammation alone.⁸⁻¹¹ Discoveries of novel T-cell subsets, notably anti-inflammatory regulatory T (Treg) cells^{12,13} and proinflammatory IL-17-secreting (T_H17)¹⁴ and invariant natural killer¹⁵ T cells, which are implicated in asthma pathogenesis on the basis of studies in animal models and limited evidence in human asthma, have added complexity to our understanding of immunoregulation. Recent studies have revived interest in mast cells,^{6,16} a subject of intense research in the 1980s and early 1990s but not widely seen as candidate targets, possibly because of limited evidence of their role in severe disease.^{6,17,18}

Most studies of asthma pathobiology to date have focused on individual or limited numbers of inflammatory cell types, making it difficult to appreciate the cell-cell interactions within complex networks that characterize inflammatory diseases, such as asthma. Therefore we have undertaken a comprehensive assessment of bronchial and circulating T cells, including

Abbreviations used

ACQ: Asthma Control Questionnaire
BAL: Bronchoalveolar lavage
BNA: Bayesian network analysis
eNO: Exhaled nitric oxide
FOXP3: Forkhead box protein 3
GINA: Global Initiative for Asthma
ICS: Inhaled corticosteroid
IQR: Interquartile range
MAIT: Mucosal-associated invariant T
Tc: Cytotoxic T
TDA: Topological data analysis
Treg: Regulatory T

mucosal-associated invariant T (MAIT)¹⁹ cells, a novel cell type not yet studied in patients with asthma or other chronic lung diseases, and relevant $T_H2/T_H1/T_H17$ cytokines, as well as mediators released in the lungs by mast cells and basophils. We have combined these pathobiologic findings with standard clinical parameters used to define asthma severity²⁰ and interrogated the rich multidimensional clinicopathobiologic data set using the novel techniques of the machine learning approach (ie, Bayesian network analysis [BNA] and topological data analysis [TDA]).^{21,22} This enabled an in-depth investigation of the roles of individual cell types in relation to asthma severity, characterization of the complex interconnectivity between the diverse clinical and pathobiologic parameters, and identification of clinicopathobiologic clusters that could point to novel asthma endotypes.

METHODS**Participants**

Eighty-four participants (18-70 years) were enrolled from the Wessex Severe Asthma Cohort, NIHR Southampton Respiratory Biomedical Research Unit, and outpatient clinics at University Hospital Southampton: 24 healthy nonatopic participants, 15 patients with mild asthma receiving β_2 -agonists alone, 23 patients with moderate asthma receiving inhaled corticosteroids (ICSs), and 22 patients with severe asthma with persistent symptoms despite high-dose ICSs ($n = 16$) and oral corticosteroids ($n = 6$, Table I and see Fig E1 in this article's Online Repository at www.jacionline.org) classified on enrollment according to previously used criteria (see Table E1 in this article's Online Repository at www.jacionline.org).¹⁵ One hundred seven participants were consented to achieve an approximate minimum group size of 15, according to the physician's assessment of severity at enrollment, which was estimated to provide 80% power at the .05 significance level to detect differences in the primary outcomes of T_H17 and MAIT cell frequencies in each tissue compartment after loss of missing data.

Study procedures

Participants were assessed based on history, examination results, skin prick test responses to common aeroallergens, spirometric results, exhaled nitric oxide (eNO) levels, serum IgE levels, and (except for patients with severe asthma) methacholine responsiveness. Lung samples were obtained by means of sputum induction,²³ bronchoalveolar lavage (BAL), and endobronchial biopsy.^{15,17} By using flow cytometry, the following T-cell subsets were characterized by their surface markers and intracellular cytokines¹⁵ in the circulation (blood) and lungs (sputum and BAL cell pellets; cells from collagenase-dispersed endobronchial biopsy specimens were used to provide bronchial mucosal cells): T_H17 -cells, T_H1 -cells, and IL-13-secreting- T_H2 cells; $CD3^+CD4^+$ (or $CD8^-$) forkhead box protein 3 (FOXP3)⁺ Treg cells;

cytotoxic T (Tc) cells (T_C1 and T_C2); $\gamma\delta$ -17 T cells; and MAIT cells. Like other authors,²⁴ we could not detect with confidence IL-4- or IL-5-secreting T cells using intracellular cytokine staining. Levels of type 1 (IFN- γ , IL-2, and IL-12p70) and type 2 (IL-4, IL-5, and IL-13) cytokines, IL-10, IL-17A, mast cell proteases, and basophil-derived basophilic granules were measured in serum, sputum, and BAL fluid by using standard methods (for details, see the Methods section, Table E2 and Fig E2 in this article's Online Repository at www.jacionline.org).

The study was approved by the Southampton and South West Hampshire Research Ethics Committee B. All participants provided informed consent.

Statistical analysis

Data were first analyzed by using standard statistical methods, classifying subjects as healthy or having mild, moderate, or severe asthma as defined above and in the Methods section in this article's Online Repository.¹⁵ BNA was then applied to all the pathobiologic and clinical features to seek association in relation to asthma severity. Finally, TDA was applied to the same data to create a network of distinct clinicopathobiologic clusters.

Data elaboration and standard statistics

Data distribution was tested by using the Shapiro-Wilk test, and data were logarithmically transformed if they were not normally distributed. For all analyses, 2-tailed P values of less than .05 were considered significant. Data were compared between the healthy and control groups (Mann-Whitney U or Student t tests) and between each asthma severity group and the control subjects (Kruskal-Wallis test or ANOVA), depending on the distribution of the data. For the latter, an overall 5% significance level was adjusted for multiple comparisons by using the Bonferroni method. Groups ranked according to disease severity were tested for linear trend by using polynomial contrasts (or the Jonckheere-Terpstra test, if not normally distributed). Data are expressed as medians with interquartile ranges (IQRs) unless stated otherwise. Correlations were tested by using the Spearman r coefficient. Kolmogorov-Smirnov tests identified significant differences between distributions within a single cluster. Data were analyzed with Prism 6.0 (GraphPad Software, San Diego, Calif) and SPSS 21.0 (IBM, Armonk, NY) software.

Network analyses

For network analyses (BNA and TDA), data were used from 62 participants with the most complete data. Missing data were imputed by using average values specific to each tissue and disease severity subgroup. A composite value was generated for each parameter by using a weighted average across each compartment: sputum and BAL fluid for concentrations of soluble mediators and blood, sputum, BAL fluid, and biopsy specimens for cell counts, providing airway and tissue composite readouts, respectively, with a matrix of 62 participants and 26 pathobiologic and 26 clinical parameters (see the Methods section and Tables E2 and E3 in this article's Online Repository at www.jacionline.org for definitions of terms). Interconnectivity between clinical and pathobiologic parameters was first explored with BNA (Genie 2.0; Decision Systems Laboratory, Pittsburgh, Pa). Data were discretized to describe nonlinear correlations into 2 bins for binary variables or 5 to 9 bins for continuous variables.

TDA

To use the full range of available clinical and pathobiologic data simultaneously to identify multidimensional features within the data set, which might not be apparent with traditional methods, we used the novel technique of TDA, which is particularly suited to complex biological data sets. This approach represents a high dimensional data set as a structured 3-dimensional network in which each "node" comprises subjects similar to each other in multiple dimensions. Lines or "edges" are drawn between nodes that contain shared data points. Statistical tests can then be performed on groups or features that emerge from the inherent structure of the data set. This method combines features of standard clustering methodologies and

TABLE I. Clinical characteristics of participants

Parameters	Healthy control subjects	Patients with mild asthma	Patients with moderate asthma	Patients with severe asthma
No.	24	15	23	22
Demographics				
Sex (M/F), no. (%)	14 (58)/10 (42)	8 (53)/7 (47)	10 (43)/13 (57)	8 (36)/14 (64)
Age (y), median (range)	28 (20-65)	26 (21-64)	36 (21-56)	53 (23-67)
Pulmonary function				
FEV ₁ (% predicted)	108 (105-113)	88 (86-103)	99 (86-107)	65 (49-82)
FEV ₁ reversibility (%)	3.3 (1.8-7.4)	13 (11-19)	10 (2.2-17)	13 (2.6-25)
PEFR (% predicted)	108 (97-116)	98 (89-107)	95 (85-100)	70 (53-82)
PEFR variability (%)	0 (0-11)	17 (10-25)	22 (17-32)	17 (12-24)
PD ₂₀ (mg methacholine)	Negative	0.19 (0.050-0.79)	0.25 (0.063-0.73)	Not done
eNO (ppb [at 50 L/s])	16 (11-21)	53 (27-107)	26 (15-51)	20 (13-38)
Clinical				
Atopy (positive skin test result, Y/N), no. (%)	0 (0)/24 (100)	15 (100)/0 (0)	20 (87)/3 (13)	15 (68)/7 (32)
No. of positive skin test results to allergens	0 (NA)	6 (4-7)	3 (2-5)	3.5 (0-5.3)
Peripheral eosinophil count (10 ⁹ /L)	0.1 (0.1-0.2)	0.2 (0.1-0.6)	0.2 (0.15-0.3)	0.2 (0.1-0.3)
Total IgE (IU/mL)	26 (10-61)	172 (21-451)	105 (35-188)	84 (31-669)
Body mass index (kg/m ²)	24.4 (22.5-28.1)	23.6 (22.7-26.5)	25.3 (23.3-30.9)	31.0 (27.1-40.9)
Smoking status				
Never smoker, no. (%)	21 (88)	14 (93)	19 (83)	17 (77)
Former smoker, no. (%) [mean pack-years]	3 (13 [4.2])	1 (7 [6.7])	4 (17 [5.8])	4 (18 [26])
Current smoker, no. (%) [mean pack-years]	0 (0)	0 (0)	0 (0)	1 (5 [49])
Duration of asthma (y)	NA	18 (15-26)	22 (9-27)	36 (21-49)
ACQ score	NA	0.60 (0.43-1.3)	1.0 (0.60-1.4)	2.8 (2.2-3.5)
GINA level of control, no. (%)				
Controlled	NA	8 (53)	5 (22)	0 (0)
Partly controlled	NA	6 (40)	15 (65)	2 (9.5)
Uncontrolled	NA	1 (6.7)	3 (13)	19 (90)
Treatment				
Inhaled steroids	No	No	Yes	Yes
Dose (equivalent µg BDP)	NA	NA	400 (200-400)	1600 (1280-2000)
Maintenance oral corticosteroids (Y/N), no. (%)	No	No	No	6 (27)/16 (73)
Mean dose if taken (mg prednisolone/d)				11
Short-acting β-agonist (Y/N), no. (%)	No	Yes	Yes	Yes
Long-acting β-agonist (Y/N), no. (%)	No	No	10 (43)/13 (57)	22 (100)/0 (0)
Leukotriene receptor antagonist (Y/N), no. (%)	No	No	1 (4)/22 (96)	15 (68)/7 (32)
Step on GINA treatment algorithm	NA	1	2-3	4-5
Inflammatory subtype, no. (%)				
Total with valid data	16	13	18	21
Neutrophilic	4 (25)	2 (15)	2 (11)	10 (48)
Eosinophilic	1 (6.3)	3 (23)	3 (17)	6 (29)
Mixed granulocytic	0 (0)	0 (0)	0 (0)	1 (4.8)
Paucigranulocytic	11 (69)	8 (62)	13 (72)	4 (19)
Sputum cell differential (%)				
Macrophages	52 (31-66)	49 (35-64)	47 (30-62)	30 (19-43)
Neutrophils	31 (11-65)	34 (22-54)	33 (16-56)	61 (32-76)
Epithelial	3.6 (2.0-24)	4.3 (1.7-10)	4.1 (1.1-21)	2.9 (0-7.8)
Eosinophils	0.38 (0-0.94)	1.5 (0.75-1.8)	0.75 (0.25-1.5)	0.69 (0-6.1)
Lymphocytes	0.1 (0-0.75)	0.3 (0-0.75)	0 (0-0.68)	0.0 (0-0.25)
BAL cell differential (%)				
Macrophages	84 (74-89)	70 (60-80)	81 (73-89)	72 (46-94)
Neutrophils	2.5 (1.0-5.9)	2.5 (1.6-4.8)	3.5 (1.8-6.4)	6.5 (1.4-29)
Epithelial cells	9.9 (3.9-18)	21 (13-35)	11 (5.6-19)	8.7 (3.3-11)
Eosinophils	0.25 (0.0-0.56)	2.0 (0.75-3.6)	1.0 (0-3.0)	0.1 (0-1.6)
Lymphocytes	1.4 (0.94-2.4)	1.5 (0.38-3.0)	1.3 (0.5-2.3)	1 (0-1.6)
Relevant comorbidities, no. (%)				
Allergic rhinitis	0 (0)	12 (80)	11 (58)	10 (46)
Nasal polyps	0 (0)	0 (0)	1 (5.3)	5 (23)
Eczema	3 (13)	7 (47)	6 (32)	4 (19)
Bronchiectasis	0 (0)	0 (0)	1 (5.3)	1 (4.5)

Values are medians with IQRs, unless stated otherwise. The inflammatory subtype is based on sputum differentials using the following cut points: neutrophilic, >61%; eosinophilic, >3%. Percentages given are derived from subjects with valid data.

BDP, Beclomethasone dipropionate; NA, not available; PEFR, peak expiratory flow rate.

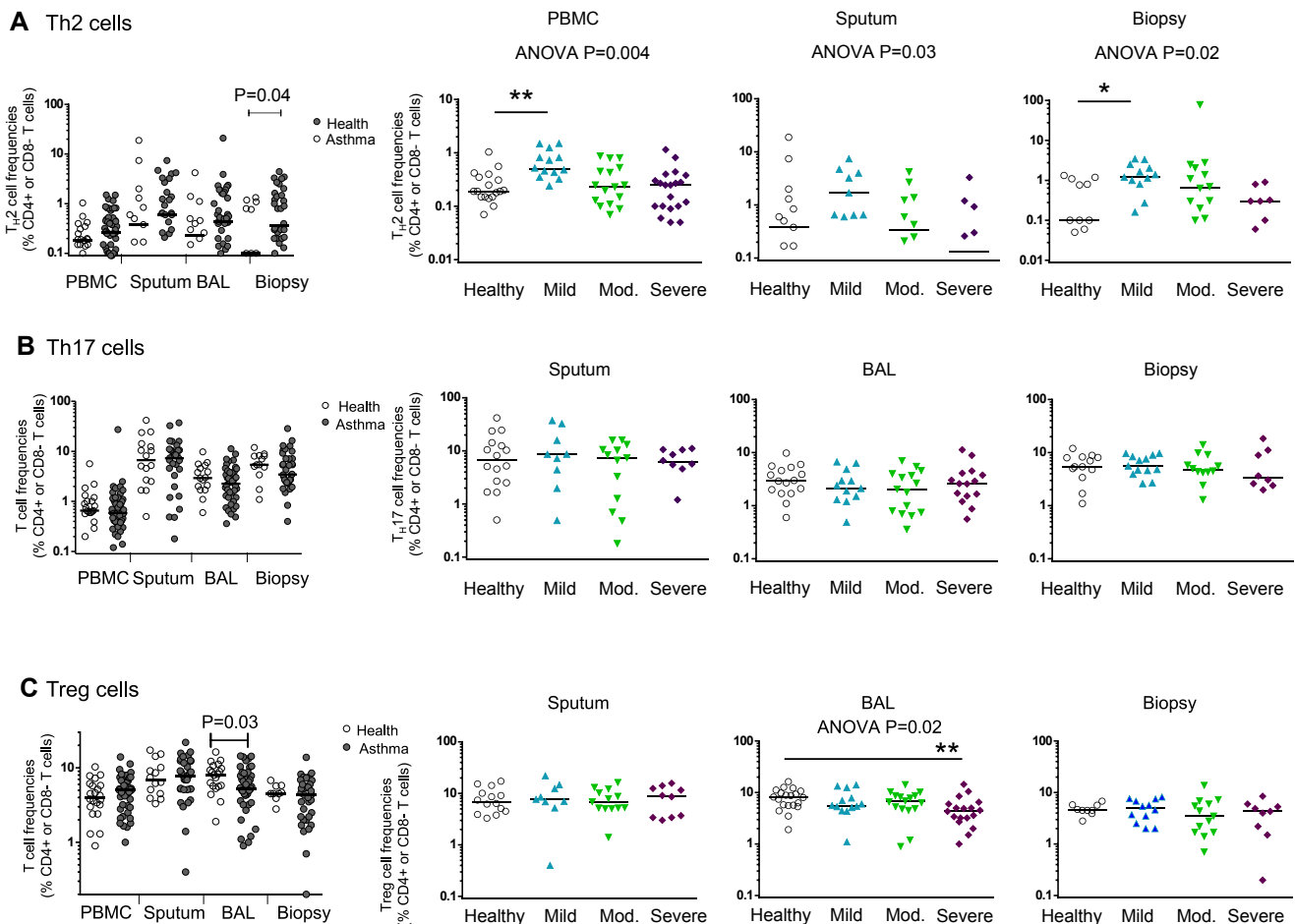


FIG 1. Frequencies of CD3⁺CD4⁺ T cells expressing IL-13 (T_H2 cells; **A**), IL-17 (T_H17 cells; **B**), and FOXP3 (Treg cells; **C**) in PBMCs, sputum, BAL fluid, and bronchial biopsy specimens as a percentage of live CD3⁺CD4⁺ T cells or, for endobronchial biopsy specimens, a percentage of CD3⁺CD8⁺ T cells. Horizontal lines show medians. Left columns, Healthy control subjects versus asthmatic patients with Mann-Whitney *U* *P* values. Right 3 columns, Stratified by disease severity with Kruskal-Wallis *P* values (*P* < .05). **P* < .05 and ***P* < .01, post hoc Dunn test compared with healthy subjects.

also provides a geometric representation of the data.^{21,22} In contrast to most other techniques that depend on prior hypotheses and that focus on pairwise relationships within the data,²³ this geometric visualization allows recognition of multidimensional features (patterns) within the data in a less supervised, data-driven manner to identify meaningful subgroups that become apparent (self-define themselves) on visualization (please see the TDA plots). In addition, TDA does not require an *a priori* definition of the number of clusters anticipated.

TDA was performed, as previously described,²¹ with IRIS 2.0 software (Ayasdi, Palo Alto, Calif), constructing networks with parameters from Table E3. Three inputs were used: a distance metric, 1 or more filter functions, and 2 resolution parameters ("resolution" and "percent overlap" or "gain"). A network of nodes with edges between them was created by using a force-directed algorithm. The nodes represent bins or "microclusters" of data points, and 2 nodes are connected if their corresponding collections of data points have a point in common.²¹ Variance-normalized Euclidean distance was used as a distance metric, with 2 filter functions: principal and secondary metric singular value decomposition (for further explanation, see the Methods section in this article's Online Repository). Resolution and gain settings were selected where the network structure permits identification of subgroups. Kolmogorov-Smirnov tests identified parameters that differentiate each subgroup from the rest of the structure and create clusters. Comparisons between multiple clusters used 1-way ANOVA, with *post hoc* tests with the Bonferroni correction.

For additional methods used, see the Methods section in this article's Online Repository.

RESULTS

Data were first analyzed by using standard statistical methods without imputation or composite averages, classifying subjects as healthy or as having mild, moderate, or severe asthma (see Table E1 and the Results section in this article's Online Repository at www.jacionline.org). Previous observations that mild steroid-naïve asthma is characterized by a bias toward type 2 inflammation were confirmed, with increased numbers of IL-13-secreting CD4⁺ (T_H2) cells in sputum, BAL fluid, and endobronchial biopsy specimens from patients with mild asthma (Fig 1, A) and ratios of IL-13- to IFN-γ-secreting CD4⁺ (T_H1) cells (see Fig E3 in this article's Online Repository at www.jacionline.org). However, this bias was not seen in patients with severe asthma, in whom frequencies of IL-13-secreting T_H2 cells were not significantly different from those in healthy subjects, although we did not measure frequencies of IL-4- or IL-5-secreting T cells. Similarly, in patients with mild asthma, there were significant increases in median concentrations of the type 2 cytokines IL-5 (median,

0.13 pg/mL; IQR, 0.05-0.19 pg/mL) compared with those in healthy subjects (0.003 pg/mL; IQR, 0.001-0.006 pg/mL; $P < .001$) and IL-13 (0.009 pg/mL; IQR, 0.0009-0.026 pg/mL) compared with those in healthy subjects (0 pg/mL [IQR, 0-0.0008 pg/mL]; $P < .05$) in BAL fluid (see Fig E4 in this article's Online Repository at www.jacionline.org). In sputum neither IL-5 nor IL-13 levels were increased in patients with mild or moderate asthma. In patients with severe asthma, IL-5 levels were also significantly increased in both BAL fluid (0.015 pg/mL; IQR, 0.007-0.19 pg/mL; $P < .05$) and sputum (6.18 pg/mL; IQR, 3.13-14.8 pg/mL) compared with levels seen in healthy subjects (1.19 pg/mL; IQR, 1.0-2.2 pg/mL; $P = .005$; see Fig E4, B), suggesting that its secretion might be relatively steroid insensitive and might be derived from cellular sources other than airway T_H2 cells.

Analysis showed no significant differences in T_H17 cell or $\gamma\delta$ -17 T-cell frequencies between asthmatic patients and healthy subjects (Fig 1, B, and see Fig E5 in this article's Online Repository at www.jacionline.org). Furthermore, we found no evidence of dysregulation of the T_H17 response during cold-induced exacerbations of asthma (see the Results section and Figs E6 and E7 in this article's Online Repository at www.jacionline.org). Likewise, IL-17 levels were not increased in BAL fluid, sputum, or serum from asthmatic patients (see Fig E4, B, and data not shown; $P > .05$ in all compartments). However, when asthma was stratified according to severity, IL-17 levels in BAL fluid were increased in patients with mild asthma ($P = .04$; see Fig E4, A). Increased IL-17 levels were associated with the presence of allergic rhinitis (0.017 pg/mL [IQR, 0.0065-0.043 pg/mL] if present and 0.005 pg/mL [IQR, 0.0033-0.010 pg/mL] if absent, $P = .02$), with airway eosinophilia ($r_s = 0.34$, $P = .04$) and high serum IgE levels ($r_s = 0.42$, $P = .007$; see Fig E4, C).

T_H17 cells share functionally and developmentally antagonistic relationships with the immunoregulatory Treg cells that regulate airway hyperresponsiveness in murine asthma models.²⁶ We observed a slight but significant deficiency of BAL $CD4^+FOXP3^+$ Treg cells in asthmatic patients (median frequency, 5.3%; IQR, 4.3% to 8.2%) compared with those in healthy subjects (8.1%; IQR, 5.6% to 10%; $P = .03$; Fig 1, C), which was restricted to patients with severe asthma (Fig 1, C) and was not evident in blood, sputum, or bronchial biopsy specimens.

There was a striking deficiency of T-cell receptor $V\alpha7.2^+CD161^+$ MAIT cells in blood, sputum, and biopsy specimens from asthmatic patients, which was related to disease severity and treatment with ICSs (Fig 2 and see Fig E8 in this article's Online Repository at www.jacionline.org). There was evidence of seasonal variation in MAIT cell frequencies and association with serum vitamin D3 concentrations and use of oral corticosteroids (see the Results section and Fig E9 in this article's Online Repository).

Application of BNA to pathobiologic and clinical features in relation to asthma severity showed complex nonlinear associations (Fig 3). Five nodes (eNO, IL-17, IFN- γ , neutrophils, and vitamin D) without strong interactions with other parameters were not connected and therefore remained outside the network. The asthma severity node was strongly connected with mast cell mediators, tryptase, chymase, and carboxypeptidase A3 and with IL-13- and IFN- γ -secreting $CD8^+$ cytotoxic T cells (T_C2 and T_C1 cells, respectively). There was a strong negative association between MAIT cell frequencies and ICS use that, in turn, was positively associated with asthma severity.

To look further for novel associations between clinical and pathobiologic features, we applied TDA to all acquired clinical and pathobiologic data (Figs 4 and 5 and see Table E4 in this article's Online Repository at www.jacionline.org). Data were treated as composite averages for T-cell subsets (across blood, sputum, BAL fluid, and biopsy specimens), cytokines and eosinophils (across blood, sputum, and BAL fluid), neutrophils, macrophages, lymphocytes, mast cell mediators, and basogranulin (across sputum and BAL fluid). One healthy and 6 asthma clusters were identified.

The TDA-derived cluster 1, comprising predominantly patients with mild atopic asthma, had (compared with other asthmatic patients) better lung function, lower Asthma Control Questionnaire (ACQ)²⁷ scores (mean, 0.88), and lower severity, as assessed by a physician on enrollment or based on Global Initiative for Asthma (GINA) criteria,²⁰ and patients were mostly not receiving ICSs. They had increased IL-13-secreting T_H2 cell numbers and lower IL-13 and tryptase levels and were predominantly paucigranulocytic (sputum neutrophils, $\leq 61\%$; eosinophils, $\leq 3\%$; Fig 4, B, and see Table E4).

Cluster 2 consisted of patients with well-controlled asthma (mean ACQ score, 0.5), with little evidence of inflammation (the only abnormality being eosinophilia) and lower frequencies of Treg cells and IFN- γ -secreting $CD8^+$ T cells (Fig 4).

Cluster 3 consisted of patients with moderately severe (defined by enrollment criteria) and partially controlled asthma (based on GINA criteria²⁰) despite ICSs; they had the highest bronchodilator reversibility and eNO levels. Their pathobiologic profile consisted of type 2 inflammation, with the highest levels of IL-5 and IL-13 and high frequencies of IL-13-secreting T_H2 cells in bronchial biopsy specimens (Fig 5, A, and see Figs E10 to E12 in this article's Online Repository at www.jacionline.org) but also other T-cell subsets, T_H1 cells, T_H17 cells, and Treg cells.

Cluster 4 was a small group with later-onset (mean, 28 years) moderately severe disease based on a physician's assessment, nasal polyposis, salicylate sensitivity, and low IL-17 levels.

Cluster 5 asthmatic patients were older (mean age, 50 years), with high BMI (mean, 32.6 kg/m²), poor lung function, high symptom scores (mean ACQ score, 2.1), and high treatment requirements (predominantly GINA step 4-5 and a mean of 1500 μ g/d beclomethasone dipropionate equivalent). Their pathobiologic profile was high type 2 cytokine levels (IL-5 and IL-13), IL-13-secreting $CD8^+$ T (T_C2) cells (see Fig E13, D, in this article's Online Repository at www.jacionline.org), and high tryptase, chymase, and carboxypeptidase A3 levels (Figs 4 and 5, C, and see Figs E10 to E12 in this article's Online Repository). However, when compared with cluster 3 (also type 2 cytokine high), cluster 5 had fewer Treg cells and higher ICS use (mean difference, 1250 μ g/d).

Cluster 6 was predominantly female, obese (mean BMI, 35 kg/m²), and nonatopic, with salicylate sensitivity and later onset (mean age, 25 years). They were the most severe cluster based on GINA classification, physician's assessment, symptom scores (mean ACQ score, 3.2), and lung function (mean prebronchodilator FEV₁, 62%), despite high-dose ICSs (see Fig E12, B, in this article's Online Repository at www.jacionline.org) and, frequently (50% of the group), maintenance oral corticosteroids (mean, 14 mg/d prednisolone). Their key pathobiologic features were high carboxypeptidase A3 levels and profound MAIT cell deficiency (Figs 4, E, and 5, D, and see Fig E9, D). They also had low T_C1 , T_H17 , and IL-13-secreting T_H2 cell numbers

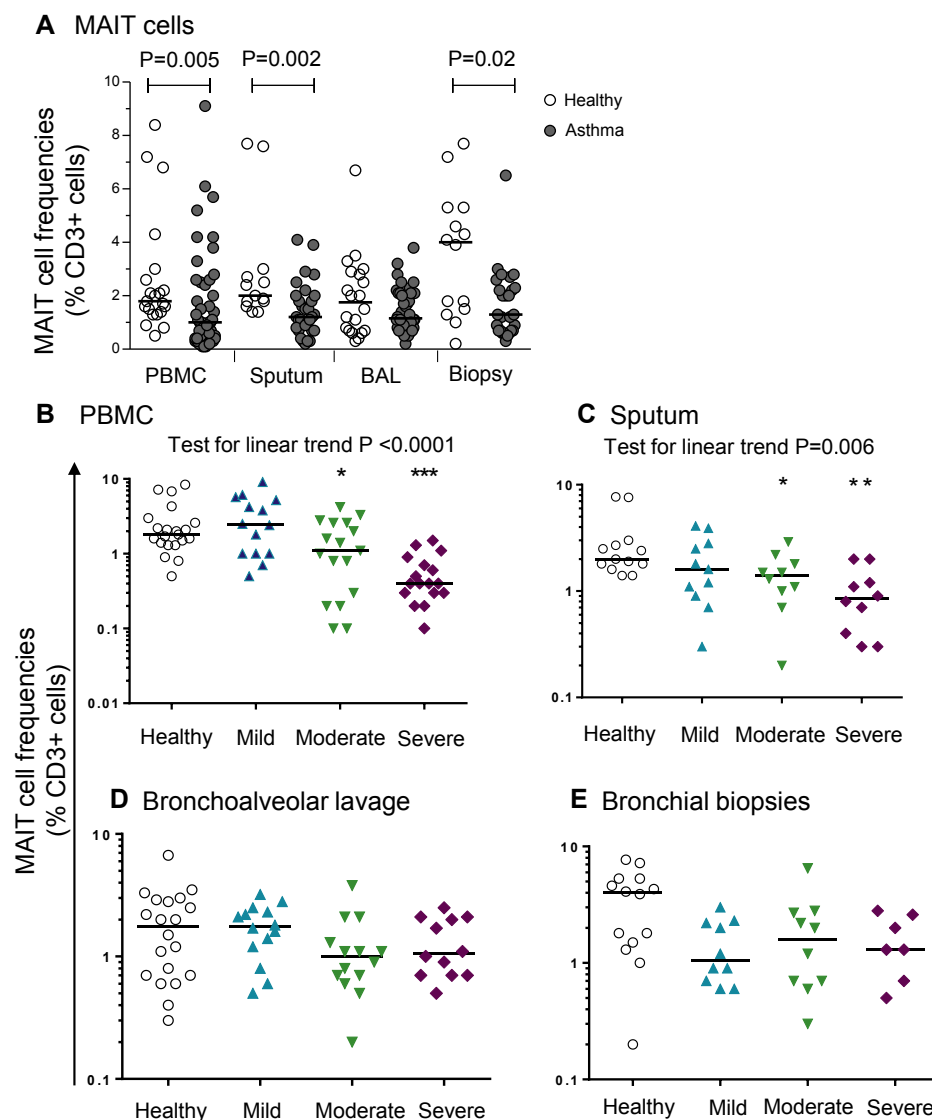


FIG 2. MAIT cells ($V\alpha 7.2^+ CD161^+$) as proportions of $CD3^+$ T cells in blood, sputum, BAL fluid, and endobronchial biopsy specimens in healthy subjects and asthmatic patients (**A**) and stratified by disease severity (**B**). Horizontal lines show medians. Unpaired *t* tests were used for log-transformed data. MAIT cell deficiency correlates with severity by linear trends across groups using residuals on log-transformed data (where $P < .05$). * $P < .05$, ** $P < .01$, and *** $P < .001$, *post hoc* Dunnett test compared with healthy subjects.

(see Fig E9, A) but increased tryptase (Figs 4, E, and 5, C) and chymase levels. This cluster contained a higher proportion of participants who could be classified as cluster 5, as described by the Severe Asthma Research Program⁹ (see Fig E13, B) and the obese, noneosinophilic cluster reported by Haldar et al.¹⁰

The majority of participants with sputum neutrophilia, which was defined as neutrophil numbers of greater than 61%,²⁸ were in clusters 5 and 6, whereas eosinophilic asthma was distributed across clusters 1, 2, 3, and 5 but not clusters 4 or 6 (Fig 4, B).

DISCUSSION

Asthma is a common disease with a clinical severity that ranges from mild forms controlled with β_2 -agonists alone or low doses of ICSs to very severe forms requiring high doses of ICSs and oral corticosteroids and, increasingly, biologics, such as the anti-IgE

mAb omalizumab.²⁹ In this study comprehensive analysis of T cells, granulocytes, cytokines, and mast cell mediators across the airway lumen, mucosa, and blood compartments pointed to their relative roles within the asthma syndrome that have not been recognized before: reduced MAIT cell frequencies as a striking feature that is related to asthma severity, reduced Treg cell frequencies in severe disease, and increased mast cell mediator levels in patients with severe disease, which is consistent with corticosteroid-insensitive mast cell activation. This study shows that the asthma spectrum can be broken down into several multi-dimensional clusters defined by combined clinical parameters and underlying mechanisms (pathobiology), which provides proof of concept for endotyping asthma for better understanding of its mechanisms and more focused drug development.

Original descriptions of asthma pathobiology³ suggested a key role for T_H2 mechanisms. Consistent with this concept,

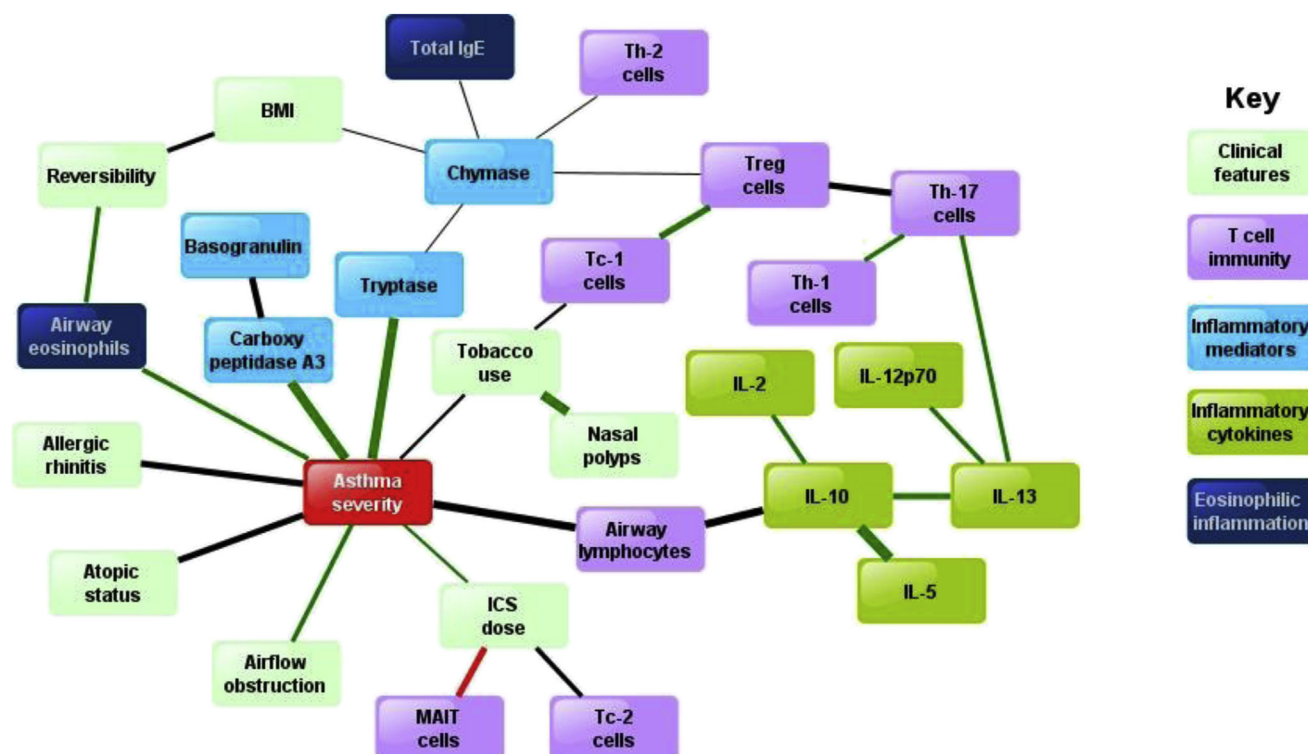


FIG 3. Bayesian belief network showing the strongest interactions between pathobiologic parameters across a range of clinical severities of asthma or health. Nodes without strong interactions are excluded. Line thickness represents strength of interaction (Euclidean distance). Line colors: *green*, positive associations; *red*, negative associations; *black*, nonlinear associations. Asthma severity is based on overall physician's assessment at enrollment (see Table E1). *BMI*, Body mass index; *T_{C1}*, CD8⁺IFN- γ ⁺ T cells; *T_{C2}*, CD8⁺IL-13⁺ T cells.

when all the asthmatic patients in this study were compared as a group with healthy participants, the most significant asthma discriminators were airway eosinophilia and higher levels of mast cell mediators (carboxypeptidase A3, chymase, and tryptase), IL-5, IL-13, eNO, and serum IgE but lower IFN- γ levels, a pathobiologic profile classically associated with T_H2 inflammation (Fig 1, A, and see Fig E11). The application of BNA showed high connectivity between the asthma severity node and nodes for mast cell mediators and IL-13- and IFN- γ -secreting CD8⁺ cytotoxic T cells (T_{C2} and T_{C1} cells, respectively) and a strong negative association between MAIT cell frequencies and asthma severity and ICS consumption. Applying the recently developed TDA method^{21,22} to the same data set showed complex multidimensional clusters (ie, possible endotypes defined by a combination of clinicopathobiologic features).^{8,11} The advantage of TDA over standard clustering methodologies is that it provides geometric representations of complex and multidimensional data sets that reveal and stratify distinct subgroups.²² It combines features of standard statistical methods, such as singular value decompositions and similarity metrics, to construct a network that clusters most similar data points into nodes. A node in a TDA network represents a group of most similar data points (in this case ≥ 2 subjects who are similar in multiple dimensions). Each node can be joined to the next node if they share common data points (ie, subjects). This allows a natural continuous network when the phenomenon is not disjointed.²¹ TDA can deal with both linear and nonlinear associations and identifies significant subgroups

in a data-driven manner, allowing for finer stratification.²¹ Furthermore, TDA is sensitive to both large- and small-scale patterns that other techniques, such as clustering and multidimensional scaling, often do not detect because they sometimes obscure geometric features captured by using topological methods. Hierarchical clustering cannot easily identify these subgroups because it tends to separate points that might in fact be close in the data.²¹

The finding of clinicopathobiologic clusters in the data set in this study should improve our understanding of asthma and inform drug development. Overexpression of the T_H2 cytokine network in cluster 1 is similar to the original reports in corticosteroid-naïve asthmatic patients, highlighting the role of type 2 mechanisms in asthmatic patients.³ Clusters 3 and 5 share many clinical features, including atopy, allergic rhinitis, and emotion-related symptoms (see this article's Online Repository). Both are characterized by type 2 inflammation, with the highest levels of IL-5, IL-13, and IL-10, suggesting that these clusters reflect asthma endotypes that might be particularly suitable for biologics, such as mepolizumab³⁰ and lebrikizumab,³¹ that currently use indirect biomarkers (ie, eosinophil counts and serum periostin levels) to select patients to maximize clinical efficacy. However, important differences between these 2 clusters were identified: higher Treg cell frequencies in cluster 3 might explain their lower corticosteroid requirements, whereas lower IL-13-secreting T_H2 cell frequencies and higher tryptase levels in cluster 5 suggest distinct, steroid-insensitive mechanisms.³² It should be noted that we stained only for IL-13, and therefore we cannot

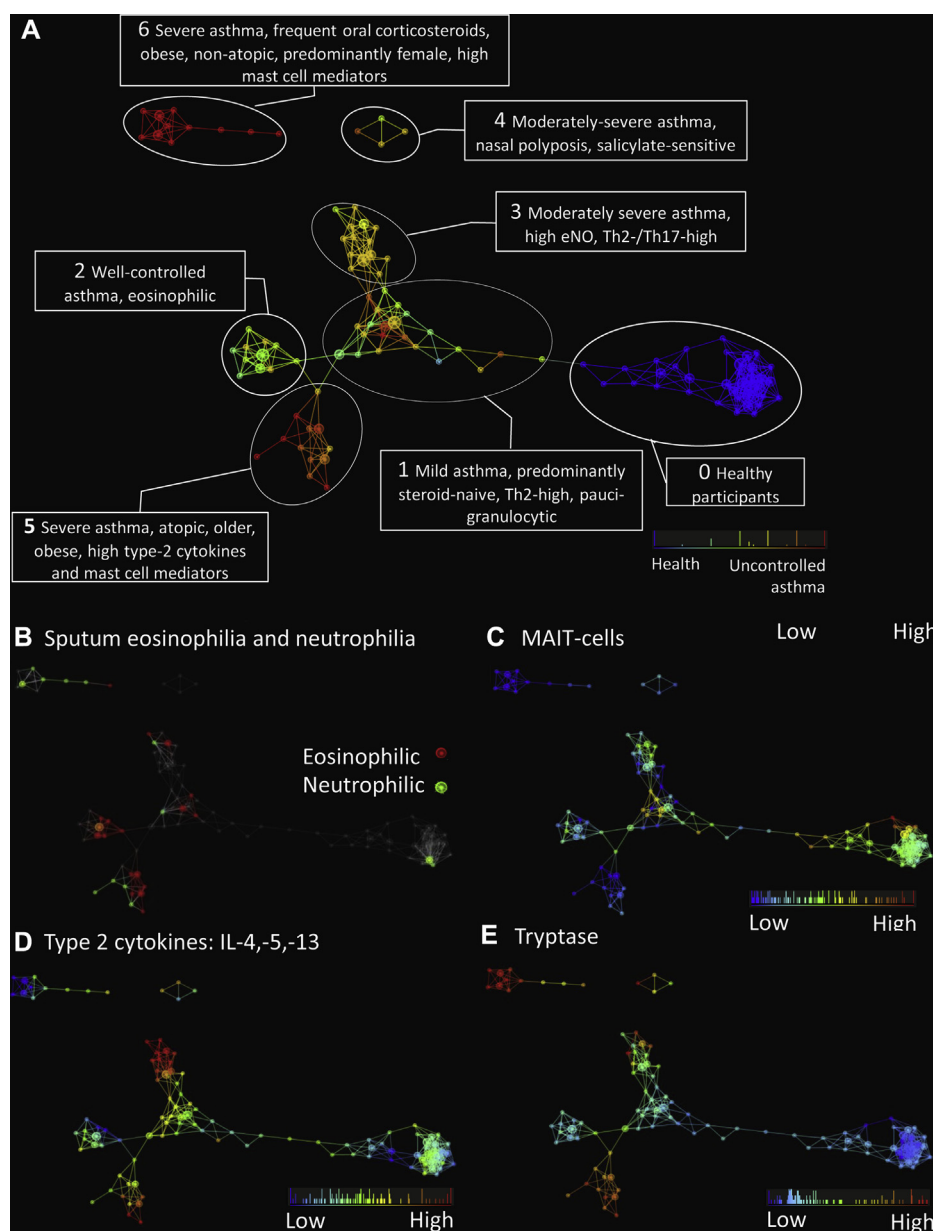


FIG 4. A, Multidimensional clinicopathobiologic clusters in asthmatic patients and healthy subjects. Topological network analysis of clinical and pathobiologic features generates 1 healthy (blue) and 6 distinct clinicopathobiologic asthma clusters (1-6). The network is colored by disease severity (GINA classification), with patients with the most severe disease in red and patients with the milder forms in varying shades of orange, yellow, and green. B, The same network as Fig 4, A, overlaid with distribution of neutrophilic (sputum neutrophils >61%, green) or eosinophilic (sputum eosinophils >3%, red) asthma. C, Frequencies of MAIT cells. D, The network is colored based on average concentrations of the type 2 cytokines IL-4, IL-5, and IL-13 in serum, sputum, and BAL fluid. E, The network is colored based on concentrations of mast cell tryptase in sputum and BAL fluid. In Fig 4, B-E, the colors represent concentrations or frequencies, ranging from low (blue) to high (red) concentrations. The TDA used 62 subjects with most complete data. The variance normalized Euclidean metric was used. The lenses used were principal and secondary singular value decomposition (resolution, 32; gain, 4.0/3.5×; equalized). Node size is proportional to the number of subjects in the node.

exclude an increase in T_H2 cells secreting IL-4 or IL-5 in the more severe asthma clusters. We observed some differences between patterns of cytokine secretion in sputum and BAL fluid, which might arise because BAL samples the distal airways and alveoli, whereas sputum measurement reflects changes in more proximal airways.³³

The strong association between asthma severity and mast cell mediator levels in clusters 5 and 6 suggests that severe asthma is a disease in which mast cell activation plays an important role. Our data add to evidence implicating mast cells in patients with severe asthma, providing additional confirmation that should stimulate the development of drugs that target mast cells. Brightling et al⁶

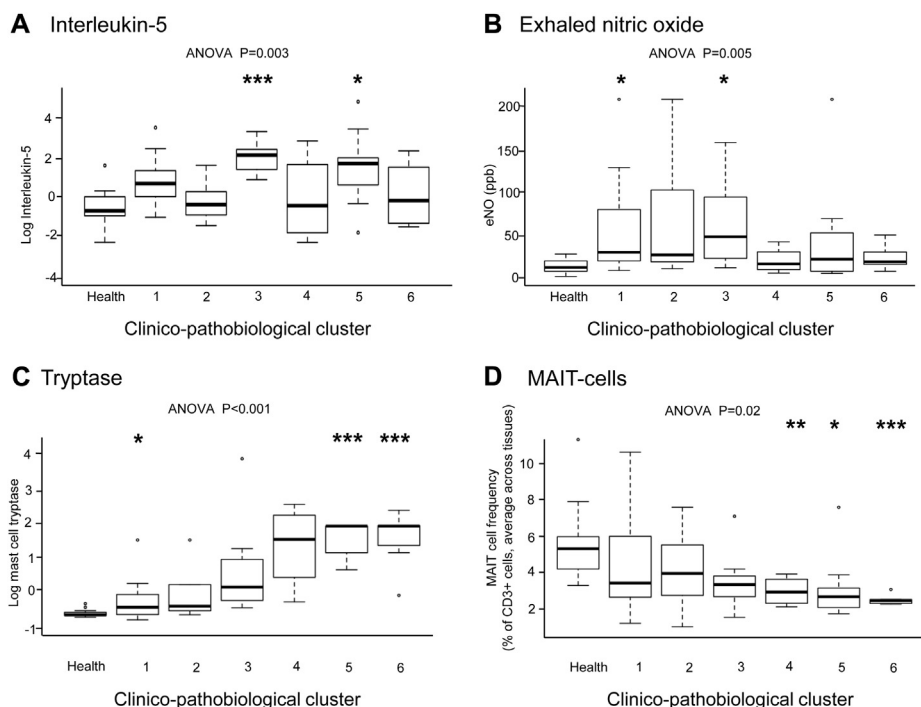


FIG 5. Analyses generated from the clinicopathobiologic TDA network in Fig 4 show concentrations of IL-5 averaged across serum, sputum, and BAL fluid (A); eNO concentrations (B); mast cell tryptase levels in BAL fluid and sputum (C); and MAIT cells in blood, sputum, BAL fluid, and bronchial biopsy specimens (D). Box and whisker plots show medians, IQRs, and ranges. Statistical tests indicate 1-way ANOVA, with *post hoc* *t* tests compared with healthy subjects by using the Bonferroni correction. * $P < .05$, ** $P < .01$, and *** $P < .001$.

have described increased numbers of tryptase-positive mast cells infiltrating the airway smooth muscle in patients with mild and severe³⁴ asthma in numbers that correlate with airway hyperresponsiveness.^{6,18} In the Severe Asthma Research Program Balzar et al¹⁶ reported that severe asthma was associated with an increase in numbers of bronchial mast cells staining positive for both tryptase and chymase and with BAL concentrations of PGD₂, a lipid mediator associated with mast cells and shown to increase after allergen challenge.⁵

The current study adds to the evidence^{5,16,35} that mast cell activation is insensitive to corticosteroids and suggests that patients with severe asthma, in whom mast cell mediator levels are increased, can be stratified further by clinical features, such as atopic status and also by evidence of type 2 cytokine-mediated mechanisms in cluster 5 but not cluster 6. We speculate that the anti-IgE antibody omalizumab might exhibit some of its beneficial effects in patients with severe atopic asthma through inhibition of IgE-mediated mast cell activation. This finding might yield a prognostic biomarker for this biologic, which is currently missing, and could extend the indication for omalizumab to non-atopic asthma, in which a preliminary trial has suggested clinical efficacy.³⁶

Several asthma studies have reported increased IL-17 levels,^{37,38} but this study found only limited evidence for T_H17 cells and none for $\gamma\delta$ -17 cells during either a period of clinical stability or an exacerbation. This is consistent with the findings of a recent trial in which the anti-IL-17 receptor A mAb brodalumab had no effect on symptoms or lung function in patients with moderate-to-severe asthma.³⁹ However, we observed associations between IL-17 concentrations and levels of traditional type 2

biomarkers (airways eosinophils and serum IgE) that have not been reported before because IL-17 has mainly been implicated in neutrophilic inflammation in asthmatic patients.⁴⁰

Our study also identified, for the first time, reduced numbers of CD3⁺CD4⁺FOXP3⁺ Treg cells in patients with severe asthma. In human subjects some upregulation of the nuclear transcription factor FOXP3 has been observed in nonsuppressive T cells on T-cell receptor stimulation.⁴¹ Although we were not able to further validate the identity of these FOXP3⁺ T_H cells as Treg cells with additional surface markers, we observed low rates of spontaneous T-cell activation, suggesting that activated T cells will comprise only a small proportion of the CD3⁺CD4⁺FOXP⁺ T cells enumerated.

An important finding in this study is the striking deficiency of MAIT cells in both the circulation and lungs, which correlated strongly with clinical severity. To our knowledge, MAIT cells have not yet been studied in any airways disease. This study suggests that they are more abundant than invariant natural killer T cells,¹⁵ comprising up to 10% of blood and airway T cells. Their marked evolutionary conservation implies an important role in immunity.^{42,43} MAIT cells are the most abundant T-cell subset able to detect and kill bacteria-infected cells. Recent animal models of bacterial airways infection indicate their critical role in lung host defense.^{42,44} We found MAIT cell frequencies to be associated with serum vitamin D3 concentrations and in pilot data could be suppressed by 1 week of treatment with prednisolone (see Fig E14 in this article's Online Repository at www.jacionline.org). The lack of a significant deficiency of MAIT cells in BAL fluid might result from low peripheral deposition of ICSSs in the more distal airways and alveolar compartments sampled by

means of lavage.³³ Their deficiency in patients with severe asthma, whether primary or resulting from chronic corticosteroid use, can contribute to increased susceptibility to bacterial infection recognized in patients with severe asthma^{45,46} and to changes in the airway microbiome⁴⁷ and might thus effect asthma pathology.⁴⁴

In summary, this study sheds light on previously unreported observations in asthma in relation to disease severity. The observation of clusters composed of clinical and pathobiologic parameters will need to be reproduced before these clusters can be accepted as novel endotypes of asthma. However, this paves the way for future asthma studies in large patient cohorts, such as the Severe Asthma Research Program⁹ and Unbiased BIOMarkers in PREDiction of respiratory disease outcomes (U-BIOPRED),⁴⁸ in which distinct asthma endotypes could be identified and subsequently validated, allowing translation to clinical trials and routine clinical practice.

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Key messages

- We provide proof of concept for a powerful new analytic approach to defining multidimensional clinical and pathobiologic clusters: TDA. This underlines the role of mast cells in 2 distinct subgroups of patients with severe asthma characterized by the presence or absence of type 2 responses.
- Evidence supporting a role for T_H17 cells in patients with severe asthma is limited.
- We describe a striking deficiency of mucosal-associated T cells, as well as a mild reduction in Treg cell numbers, in patients with severe asthma.

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Title: Innate and adaptive T cells in asthma: relationship to severity and disease mechanisms

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Supplementary information on methods

Preparation of airway samples

Sputum induction was performed according to the recommendations of the European Respiratory Society¹ and the samples were immediately processed with dithioerythritol to separate cells from the fluid phase of the sputum, as previously described². Fibreoptic bronchoscopy for bronchoalveolar lavage (BAL) and bronchial biopsy was performed in accordance with the recommendations of the American Thoracic Society.³ For flow cytometry cells were processed either immediately (for analysis of MAIT-cells) or cultured overnight for analysis of other subsets. Prior to antibody staining, bronchial biopsies were dispersed with collagenase I (Sigma) reconstituted in RPMI 1640 without L-glutamine (Sigma) at 1 mg/ml for 1 hour at 37°C.²

Flow cytometry

MAIT-cells were identified in peripheral blood mononuclear cells (PBMC), sputum, BAL and biopsies by flow cytometry for surface markers, using a nine-color FACS AriaTM cell sorter (BD Biosciences, Oxford, UK). Live cells were identified using LIVE/DEAD® Fixable Violet Dead Cell Stain for 405 nm excitation (L34955, Invitrogen) according to the manufacturer's instructions. Cells were resuspended and washed in fluorescence activated cell sorting (FACS) buffer (0.5% (w/v) Bovine Serum Albumin, 2 mM EDTA in Phosphate Buffered Saline [pH 7.4]) then stained with fluorochrome conjugated antibodies in 110 µl FACS buffer for 30 min at 4°C. Doublets were excluded using pulse-width and pulse-area and side scatter properties.² MAIT-cells were identified by the following monoclonal antibodies in combination with anti-CD3, anti-CD4, anti-CD8 monoclonal antibodies (BD Biosciences): PE-conjugated anti-Vα7.2 (clone 3C10, Biolegend, San Diego, CA)

and FITC-conjugated anti-CD161 monoclonal antibody (BD Biosciences). $\gamma\delta$ T-cells were identified by anti- $\gamma\delta$ TCR-FITC (BD Biosciences).

For intracellular cytokine analysis that allowed identification of Th17, Th1 and IL-13-secreting Th2 T-cells, whole bronchial biopsies were cultured overnight in AIM V® serum free medium (Gibco, Life Technologies, Paisley, UK) supplemented with: 0.5 μ g/ml Fungizone, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μ g/ml streptomycin, 100 U/ml penicillin (all Gibco) and 0.004% (v/v) 2-mercaptoethanol (Agilent Technologies UK, Stockport, UK) at 37°C in the presence of 5% CO₂, as previously described by our group.² Cell aliquots obtained by sputum induction or bronchoscopy, were resuspended in AIM V® at a concentration of 1x10⁶ cells/ml, and cultured overnight in the same manner. The following day, samples were stimulated for 5 h with 25 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin (both Sigma, Gillingham, UK) in the presence of 2 μ M monensin (eBioscience, Hatfield, UK). Paired samples were left unstimulated, without PMA, ionomycin or monensin, as negative controls and for FOXP3 staining. Bronchial biopsies were then dispersed with collagenase I. Cells were stained using 1 μ l LIVE/DEAD® Fixable Violet for 30 min, washed again and fix-permeabilized with eBioscience fixation/permeabilization reagents according to the manufacturer's instructions. CD4⁺ and CD8⁺ T-cell subsets were identified by intracellular staining for 45 min with the following monoclonal antibodies: anti-IL-13-FITC (R&D Systems, Abingdon, UK), anti-IL-17A-PE, anti-interferon(IFN)- γ -allophycocyanin (APC), anti-Tumor Necrosis Factor (TNF)- α -APC, anti-FOXP3-APC (all eBioscience), followed by cytometry as described above. Representative flow cytometry plots are presented in Figure E2.

Confirmation of MAIT-cell receptor sequence

In order to validate whether cells identified by dual staining for TCR-V α 7.2 and CD161 were indeed MAIT cells, the identity of the TCR was confirmed by PCR. Briefly, live CD3+V α 7.2+ CD161+ cells were sorted directly *ex vivo* from peripheral blood into sorting media and cell lines (clones) established. After 2-3 weeks culture *ex vivo* these clones were again stained and V α 7.2+CD161+ cells were sorted. RNA was extracted from using TRIzol® LS Reagent (LifeTechnologies) and chloroform (Sigma), reverse transcribed using SuperScript™ III reverse transcriptase (Invitrogen, Paisley, UK) and amplified using reverse-transcription polymerase chain reaction performed using BioTaq DNA Polymerase (Gentaur, Brussels, Belgium). The reaction mixture, 50 μ l total, contained 1 μ l of cDNA, 5 μ l 10x NH₄ buffer, 2.5 μ l of 50 mM MgCl₂, 1 μ l of 10mM dNTP, 0.5 μ l of BioTaq DNA polymerase, 1 μ l of forward primer, 1 μ l of reverse primer and 39 μ l of ddH₂O. Amplifications were performed on the Mastercycler (Eppendorf, Stevenage, UK) with the following cycling parameters: 96 °C for 1 minute pre-incubation, then 96 °C for 30 s denaturing, then 65 °C for 30 s annealing, then 72 °C for 2 min elongation; repeated for 45 cycles and finished with a 10 min extension at 72 °C. Primers (MWG Biotech AG, Ebersberg, Germany) were designed to span the canonical V α 7.2-J α 33 rearrangement of the CDR3 α region of the TCR α chain.⁴

1. TCR V α 7.2

5'-ATA TAT CAT ATG GGA CAA AAC ATT GAC CAG-3' forward

2. TCR J α 33

5'-GCT TTA TAA TTA GCT TGG TCC CAG C-3' reverse

10 µl of reaction product premixed with dye were loaded into wells of a 1% (w/v) agarose (Melford, Chelsworth, UK) gel in Tris/Borate/EDTA buffer containing 10 µl per 100 ml of Nancy-520 dye (Sigma) and electrophoresed at 80 volts for 60 min, before imaging under ultraviolet light. A clear product of appropriate length was identified in these clones and in a positive control (Jα33 cDNA) but not in a non-MAIT iNKT clone (negative control), confirming that cells identified according to their surface staining did indeed express the canonical Vα7.2-Jα33 TCR rearrangement.

Measurement of mast cell proteases

Various combinations of polyclonal and monoclonal antibodies prepared against tryptase, chymase or carboxypeptidase A3 were investigated in a sandwich ELISA development. Immunoglobulins were purified from hybridoma culture supernatants by ammonium sulphate precipitation⁵ followed by protein G affinity chromatography (GE healthcare). For use as detecting antibodies immunoglobulins were biotinylated by biotinamidocaproate N-hydroxysuccinimide ester (Sigma).

The ELISA for tryptase was performed as previously described,⁵ with rabbit antiserum as coating antibody and monoclonal antibody AA5 for detection. Proteases purified from human skin⁶ were employed as assay standards. Chymase and carboxypeptidase A3 were measured by sandwich ELISA using monoclonal antibodies developed in-house.

Measurement of basogranulin

Basogranulin levels were determined by a dot blotting procedure with specific monoclonal antibody BB1 essentially as described previously.⁷ A lysate of peripheral blood basophils purified using MACS Basophil Isolation Kit (Milteny Biotec, Bisley, UK) served as the standard.

Measurement of cytokines

Cytokines were measured by multiplex enzyme-linked electrochemiluminescent assays (Meso Scale Discovery, Gaithersburg, MD) in serum, sputum supernatant and BAL. For cytokine assays of BAL samples, BAL supernatants were first concentrated 45-fold by centrifugal dialysis using 'Vivaspin 6' concentrators (Sartorius Stedim Biotech GmbH, Göttingen, Germany) at 2000 g for 4.5 h at 4°C.

Topological data analysis

TDA was performed using variance normalized Euclidean distance as a distance-metric. The Normalized Euclidean distance between two points takes into account that each column in the data set could have significantly different variance. The Normalized Euclidean (or Variance Normalized Euclidean, VNE) distance between two points X and Y is given by:

$$VNE(X, Y) = \sqrt{\sum_{i=1}^N \frac{(X_i - Y_i)^2}{V_i}}$$

Where V_i is the variance associated with each column i and is given by

$$V_i = \frac{1}{M} \sum_{j=1}^M (Z_{j,i} - \bar{Z}_i)^2$$

And $\bar{Z}_i$ is the mean of column i and is given by

$$\bar{Z}_i = \frac{1}{M} \sum_j Z_{j,i}$$

TDA also used two filter functions: principal and secondary metric singular value decomposition. These filter lenses maps data into a Euclidean space using the rows

of the distance matrix as the coordinates and then performs principal component analysis. The data is subsequently projected via multi-dimensional scaling (MDS) by finding an embedding for X, Z :

$$f(\mathbf{X}) = \min_{\mathbf{Z}} \sum_{i,j} (d(X_i, X_j) - L_2(Z_i, Z_j))^2$$

Data elaboration for TDA

TDA analyses used data from the 62 participants with the most complete datasets. A wide range of parameters, in multiple compartments (blood, sputum, BAL and bronchial biopsies), some requiring invasive testing, were acquired which meant that nearly 16% of data were missing values. However, as each cytokine, mediator or T-cell subset had been measured in paired compartment samples from each participant, it was possible to minimize the impact of missing data by generating for each parameter a composite average metric across each tissue. Composite variables have been used by others⁸ and were necessary as both Bayesian network analysis and TDA are sensitive to missing data, with potential to create subgroups that are an artefact, to an extent determined by the fact that there are many missing values.

First, missing data were imputed for each individual parameter using the average value specific to each compartment and disease severity subgroup, the severity being defined by clinical and physiologic criteria defined before the study began (see table E1). Data were normalized by dividing each parameter by the group mean and a single composite average value was then generated for each parameter using the average across each compartment, thereby creating a matrix of 62 participants and

52 rows (26 pathobiologic parameters, 26 clinical parameters). Composite averages were obtained for T-cell subsets (from blood, sputum, BAL and biopsies), cytokines, eosinophils (from blood, sputum, BAL), neutrophils, macrophages, lymphocytes, mast cell mediators, basogranulin (from sputum and BAL). Comparisons between multiple groups used one-way analysis of variance (ANOVA), followed by post-hoc *t* tests with Bonferroni correction for multiple comparisons: using as reference category the healthy controls. Composite variables used in the TDA are defined in Table E2. Parameters which are used to generate the network are listed in Table E3.

Throughout the main paper, results presented are only for these composite averages. However, it was also possible to perform additional analyses for each parameter according to each specific tissue using the full dataset from all 84 participants without the need for any imputation. This approach has the advantage of determining how processes differ between distinct tissue compartments. For instance in comparison with BAL, sputum is considered as more representative of processes occurring in the more proximal rather than distal airways. These analyses are presented in this supplementary appendix in addition to further analyses using composite variables (see the results section below).

Analysis of seasonal variation of MAIT-cells

A cyclical pattern throughout the entire period during which the study was performed was suspected to explain the variations of MAIT-cell frequencies over time; this was analyzed using non-linear regression on log-transformed data using a standard sine function, as follows:

$$\text{Ln (MAIT frequency)} = 0.0795 + 0.6024 \cdot \sin ((2\pi \cdot [\text{Seasonal quarter}]/4) + 78.27)$$

Likewise a cyclical pattern in serum vitamin D3 concentrations was analyzed as follows:

$$\text{Ln (vitamin D3)} = 3.865 + 0.4804 \cdot \sin((2\pi \cdot [\text{Seasonal quarter}]/4) + 78.27)$$

The quarters began on 1st January and ended on 31st December.

Supplementary Results

Presentation of clinical classification in the main text and the supplementary appendix

Initial clinical classification of all recruited participants was performed using the criteria agreed by the authors prior to the start of the analysis (see Table E1). All participants were stratified as healthy, mild (steroid-naïve), moderate or severe based on the criteria applied at enrolment. Participants with asthma were also classified by the GINA criteria⁹ and characterized further using the Asthma Control Questionnaire¹⁰ and the GINA control criteria⁹ to enable readers to interpret the results of this study in relation to widely used classification methods.

The application of TDA to the combined clinical and pathobiologic parameters created 6 multi-dimensional clinico-pathobiologic asthma clusters which are named as cluster-1 to cluster-6, in increasing order of overall clinical severity according to GINA criteria.

Further analysis of data from the complete cohort without imputation

IL-17 has usually been hypothesized to be implicated in neutrophilic inflammation in asthma.¹¹⁻¹³ However we observed an increase in IL-17 in a subset of patients with mild, steroid naïve asthma, which did not correlate with an increase in Th17 cell

frequencies. An alternative cellular source of IL-17 might be the respiratory epithelium. Consistent with this, it was noted that BAL IL-17 concentrations were elevated exclusively in participants in whom the same BAL contained sheets of bronchial epithelium. When participants were dichotomized by the upper limit of normal range (97.5th percentile in health) for BAL epithelial cell frequencies, median BAL IL-17 concentrations are 4.0 fold higher in participants with abnormally high epithelial cell counts (0.023 pg/ml (IQR, 0.018-0.076)) than other participants (0.0057 (IQR, 0.0024-0.0090), $P < 0.001$) (Figure E4C). IL-17 has been implicated in inflammation of the upper airway epithelium.¹⁴ Inflammation of the lower airway epithelium is known to cause loss of epithelial integrity,¹⁵ due to disruption of tight junctions which may be promoted by IL-17.¹⁶ Therefore BAL IL-17 may be associated with epithelial fragility.

Longitudinal cohort

Whilst our data suggest that Th17 cells did not play a role in stable asthma, we considered the possibility there might be a dysregulation of the dynamics of the Th17/Treg response during acute, viral exacerbations of asthma, which are known to be associated with neutrophilic inflammation.¹⁷ We therefore measured the dynamics of the Th17, Th1, IL-13-secreting Th2 and Treg subsets in peripheral blood and sputum in 26 frequently-exacerbating asthmatics during a naturally occurring upper respiratory tract infection. We observed no significant changes in T cell frequencies in peripheral blood, and only a 1.8-fold increase in mean sputum Th17 cells from 6.2% at baseline to 11% at symptom day 7 ($P = 0.034$) (Figure E6). Furthermore we observed no significant difference in the area under of curves of PBMC Th17 response between subjects whose upper respiratory tract infection was complicated by an acute asthma exacerbation (mean 12.5, (95%CI, 7.95-17.2)) and those where

it was not (mean 7.59, (95%CI, 3.93-11.2), $P=0.2$, Figure E7). Together these data suggest that Th17 and $\gamma\delta$ T-17 cells do not play a significant role in human asthma.

MAIT-cells are deficient in asthma, modulated by corticosteroids and associated with vitamin D levels.

Having observed a deficiency of TCR V α 7.2⁺ CD161⁺ mucosal associated invariant T (MAIT) cells in asthma we sought to elucidate potential causes. We first considered whether this might be related to age, in view of the slightly higher age of the participants with more severe asthma. An additional cohort of older healthy control participants (median age of 53 years, IQR 48-57, $n=12$) was, therefore, recruited and analysis of blood MAIT-cell frequencies showed no difference in this additional cohort when compared with younger healthy participants (median age 27, IQR 24-34, $n=15$, $P=0.4$) (data not shown).

Next we considered whether this deficiency might be related to the therapeutic use of corticosteroids and, therefore, performed two open-label studies with inhaled and oral corticosteroids. No significant change in MAIT-cell frequencies was seen in blood or sputum of 12 steroid-naïve mild asthmatic participants after a 7-day course of low dose (200 micrograms twice daily) of ultrafine particle beclometasone dipropionate (Qvar) (data not shown). By contrast, there was a significant ($P=0.03$), 23% decrease in median peripheral blood MAIT-cell frequencies after 7 days of prednisolone given at a dose of 20 milligrams once daily (median 1.63% (1.1-4.4%) pre-treatment compared with median 1.26% (0.92-1.9%) post treatment)(Figure E14A). Furthermore, this steroid-induced modulation of T-cell frequencies was specific to MAIT-cells as it was not observed with a non-MAIT T-cell subset (median 1.91% (1.4-2.2%) pre-treatment compared with median 1.88% (1.5-2.3%) post treatment, $P>0.05$, Figure E14B). This suggests that the observed MAIT-cell

deficiency may have been caused by chronic use of corticosteroids, but further in-depth studies are required to confirm and elucidate further the nature of this apparent cause-effect relationship.

Murine studies have suggested that vitamin D3 is important for the development and function of iNKT-cells,¹⁸ and this has been linked to experimental airways hyper-reactivity (AHR) in mice.¹⁹ Therefore, we sought to identify first whether there was a seasonal pattern in MAIT-cell frequencies given the known seasonal variation in vitamin D levels.²⁰ This showed a highly significant association between blood MAIT-cell frequencies and the season in which blood samples were taken (ANOVA on log-transformed data, $P < 0.001$), with peak frequencies in August and a nadir in February (Figure E14C), consistent with an association with sunlight exposure. Regression analysis suggested that seasonal variation explained 16% of the variance in MAIT-cell frequencies ($R^2 = 0.16$). This effect was specific to MAIT-cells as it was not observed with a non-MAIT-cell population (V α 7.2+CD161- cells, ANOVA $P > 0.05$, data not shown) and was not due to corticosteroid therapy as it was observed in both the healthy and mild-asthmatic participants who were not on corticosteroids. We then completed the investigation by measuring serum vitamin D3 concentrations in serum samples paired with the blood MAIT-cell samples using mass spectrometry. This showed significant association (Pearson's $R^2 = 0.28$, $P = 0.01$) between serum vitamin D and blood MAIT-cell frequencies, and regression analysis showed that 28% of the variability in MAIT-cell frequencies is attributable to variations in serum vitamin D3 (Figure E14D, ANOVA $P < 0.0001$ with *post hoc* Dunnett's *** $P < 0.001$ when compared with January-March).

Analysis of mast cell mediators stratified by physician assessment of severity

Data on airway mast cell mediators are presented in the main text stratified according to the clusters identified by TDA. In addition, data from the full dataset are presented in Figure E11, comparing asthma with health (Figure E11 A-C) and stratified according to physician assessment of severity at baseline (Figure E11 D-F). In asthma there was a significant increase in the mast cell mediators tryptase (median 5.4 ng/ml (4.6-9.4) in asthma, median 4.3 ng/ml (4.0-4.8) in health, $P=0.003$) and carboxypeptidase A3 (median 0.35 ng/ml (0.065-1.0) in asthma, median 0.057 ng/ml (0.016-0.097) in health, $P=0.001$), but not chymase (median 2.4 ng/ml (0.0-5.4) in asthma, median 1.1 ng/ml (0.0-3.1) in health, $P=0.4$) in bronchoalveolar lavage, compared with health. When stratified by disease severity carboxypeptidase was significantly elevated in mild and severe groups, with a similar, but non-significant tendency in the moderate group. Likewise carboxypeptidase was significantly elevated in the mild and moderate groups with a similar but non-significant tendency in the severe group. By contrast in sputum asthma was associated with a significant increase only in chymase (median 1.4 ng/ml (0.0-10) in asthma, median 0.0 ng/ml (0.0-0.46) in health, $P=0.04$), and this was significant for the severe group.

Additional results from TDA applied to clinico-pathobiologic parameters

In addition to the results presented in the main paper, a number of additional features are apparent within the dataset. It is important to note that, after ANOVA, *post-hoc* analysis used a very conservative approach to correct for multiple comparisons, applying Bonferroni correction. Nevertheless, these analyses should be seen as exploratory, to be seen as hypothesis generating rather than hypothesis testing.

Figures E9A and B provide evidence that type-2 inflammation (as defined by IL-13-secreting Th2-cells, and the cytokines IL-5 and IL-13) is not a uniform feature of asthma. Flow cytometry revealed the highest frequencies of Th2-cells in the bronchial biopsy tissue from cluster-3 (Figure E9A). The type-2 cytokine, IL-13 (composite average across serum, sputum and BAL) was also most elevated in cluster-3, but was also significantly elevated compared cluster-5. As the asthmatics in cluster-5 were on high dose ICS (mean 1500 mcg bdp equivalent) and had low Th2 frequencies, this suggests that IL-5 and IL-13 were from a different cellular origin and that the mechanisms regulating their production are steroid-insensitive. Possible sources include innate lymphoid cells,²¹ IL-13 secreting CD8+ (Tc2) cells, mast cells, basophils, eosinophils^{22,23} and macrophages.²⁴

The doses of inhaled corticosteroid (ICS) were higher in the severe cluster-5 and cluster-6 (Figure E12). Long-term maintenance oral corticosteroid use was almost exclusive to group F, with a median dose of 14 mg/day oral prednisolone in the 50% of participants who were taking this drug.

Additional differences amongst clusters are shown in Table E4. Atopy (defined as a positive skin prick test to at least 1 common aeroallergen) was common in clusters 1,3,4 and 5, but significantly less common in cluster 6 ($P=0.01$). Likewise, allergic rhinitis was less common in cluster-4 and cluster-6, whilst nasal polyposis was observed only in the three more severe clusters-4 to 6. As groups 3 and 5 had in common the pathobiologic feature of high type-2 cytokines – the highest levels of IL-5, IL-13 and IL-10 – these groups were compared. When compared with other asthmatic clusters, both cluster-3 and cluster-5 had higher rates of atopy ($P<0.05$), and symptoms triggered by exercise ($P<0.05$) or by emotion ($P<0.001$). However,

cluster-5 differed from cluster-3 at a pathobiologic level as group 5 had significantly higher tryptase ($P=0.02$) and lower frequencies of Treg ($P=0.02$).

Evidence suggests that mast cells may respond to stimuli such as changes in the osmotic environment associated with exercise. We, therefore, compared pathobiologic features in participants with and without exercise induced asthma. We observed that the only pathobiologic feature associated with exercise-induced asthma was elevated levels of tryptase (composite from BAL and sputum, KS $P=0.004$), with a similar trend for carboxypeptidase A3 ($P=0.052$) but not for chymase (data not shown).

Comparison of TDA with clusters identified by other authors

In order to compare how the clinico-pathobiologic clusters in the current study compare with previously reported endotypes, each participant in the current study was classified according to either the hierarchical decision tree used by the Severe Asthma Research Program (SARP)⁸ or by a best fit approach to the endotypes described by Haldar *et al*²⁵ and Lötvall *et al*²⁶ and then mapped these on to the TDA network. In general there was no close agreement with descriptions by others, which may not be surprising as these had focused primarily on clinical features.

In our topological analysis the location of each subject within the network is determined to some extent by every single clinical and pathobiologic parameter; the relative influence of each being represented within Table E4 by the KS scores. Nonetheless, the formation of some clusters appeared to be influenced predominantly by homogeneous clinical features, such as cluster-2 – significantly characterized by low symptomatology (see Table E4) without any dominant influence from a single immunological parameter. Conversely other clusters were influenced

more by a constellation of pathobiologic parameters. For instance cluster-3 is distinguished by higher frequencies of T cell subsets or higher concentrations of T cell cytokines, implying perhaps a distinct pathobiologic process of T cell activation underlying this cluster. The composition of the other clusters is in each case driven by a mixture of interacting clinical and pathobiologic parameters.

Nonetheless, participants in the clinico-pathobiologic group 6 were significantly more likely (75% of group) than other participants (15% of group) to be classified by SARP as 'cluster 5', i.e. severe, later onset, female predominant (t test $P=0.002$)(Figure E13B). Similarly, participants in cluster-6 tended to be in the 'obese non-eosinophilic' cluster described by Haldar, although this association did not reach statistical significance (63% of group v 26%, t test $P=0.054$). Lötvall *et al.* specified several other putative clusters from their literature review,²⁶ which included 'severe, late onset, hypereosinophilic asthma', and 'aspirin-sensitive asthma'; the latter cluster mapped on to cluster 4 and cluster-6 (both 50% of group v 11% t test $P=0.005$) (Figure E13C).

Figure Legends

Figure E1. Flow diagram showing study design and recruitment.

Figure E2. Representative flow cytometry plots

Typical flow cytometry plots for T-helper cell subsets measured by intracellular cytokine staining. Doublets were excluded by gating on forward scatter-area (pulse area) versus forward scatter-width (pulse-width). Dead cells were excluded by their increased uptake of Violet LIVE/DEAD® Fixable. T cells were identified by side scatter profile and surface staining for CD3-PE-Cy7. T helper cells were identified by staining for CD4-PerCP-Cy5.5 (PBMC, BAL, sputum) or by lack of staining for CD8-APC-Cy7 (bronchial biopsies). Gates for IL-17, IFN- γ and IL-13 were set on controls comprising unstimulated cells using the same stains. Treg were identified by intracellular staining for FoxP3. As FOXP3 is not significantly expressed in CD4-cells this gate was set by the level amongst CD4-ve cells, such that the P1 gate contained $\leq 0.5\%$ of CD3+ cells, as shown.

Figure E3. Major CD4+ T cell subsets stratified by disease severity

(a) Ratios of CD3+4+ T cells expressing IL-13 (Th2 cells), to those expressing IFN- γ (Th1 cells) and (b) frequencies of CD3+4+ T cells expressing IFN- γ (Th1 cells) in PBMC, sputum, BAL and bronchial biopsies measured by intracellular cytokine staining and flow cytometry. Results are expressed as a percentage of live CD3+CD4+ T cells, or for bronchial biopsies, as a percentage of CD3+8- T cells. Medians are shown as horizontal lines. Left hand column of panels shows healthy

controls compared with asthmatics, and P values are from Mann-Whitney U tests. Right three columns of panels show the same data stratified by disease severity (defined at enrolment) where P values are from Kruskal-Wallis tests and are given where $P < 0.05$. Significance post hoc by Dunn's compared with health: * $P < 0.05$, ** $P < 0.01$. Abbreviations: BAL, bronchoalveolar-lavage, PBMC, peripheral blood mononuclear cell.

Figure E4. T-cell cytokines in bronchoalveolar lavage fluid and sputum supernatant

Cytokines were measured in duplicate in bronchoalveolar lavage (BAL) (A) and sputum (B) from 60 individuals (18 healthy controls, 12 mild, 16 moderate and 14 severe asthmatics). (C) BAL IL-17 was associated with allergic rhinitis, airways eosinophilia, serum IgE and the extent of epithelial shedding (see results in supplementary appendix for the method used to assess shedding). Cytokines were measured by multiplex enzyme-linked electrochemiluminescent assay using the Meso Scale Discovery platform. BAL samples were first concentrated 45-fold by centrifugal dialysis. Log-transformed data were compared by ANOVA (where significant, the P value is given at the top of each figure). Significance *post -hoc* by Dunn's compared with health: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Abbreviations: BAL, bronchoalveolar-lavage; LOD (---), limit of detection; r_s , Spearman's correlation coefficient.

Figure E5 IL-17 secreting $\gamma\delta$ T cells in health and asthma in peripheral blood and bronchoalveolar lavage

Frequencies of $\gamma\delta$ T cells secreting IL-17 as a proportion of total $\gamma\delta$ T cells in peripheral blood and in bronchoalveolar lavage measured by intracellular cytokine staining and flow cytometry. Groups are compared by Mann-Whitney (two groups) or Kruskal-Wallis tests (multiple groups). Frequencies of $\gamma\delta$ T cells were too low for accurate assessment in sputum or bronchial biopsies. Abbreviations: BAL, bronchoalveolar-lavage, PBMC, peripheral blood mononuclear cell.

Figure E6 T cell frequencies in peripheral blood and sputum during acute viral infection-induced exacerbations.

Frequencies of CD4⁺ T cells in (A) peripheral blood and (B) induced sputum during exacerbations caused by acute upper respiratory tract infections measured by intracellular cytokine staining and flow cytometry on samples processed directly *ex vivo*. Day 0, baseline screening visit (n=14); day 4, symptom day 4 (n=13); day 7, symptom day 7 (n=14). Plots show means $\pm$ 95% confidence intervals. In general data are not paired. Mean frequencies of sputum Th17 cells increased significantly 1.8-fold from 6.2% at baseline to 11% at symptom day 7. (C) T cell frequencies in paired samples of cryopreserved peripheral blood taken from 26 individuals at day 0, baseline screening visit, at day 1 and days 4, 7, 10, 13, 17 (each $\pm$ 1 day) and day 30-35, showing the dynamics of the T cell response with greater temporal resolution.

Figure E7 Peripheral blood Th17 response according to whether experienced an exacerbation.

(A) Peripheral blood Th17 cell frequencies measured by flow cytometry on cryopreserved samples plotted over time and stratified by whether subjects experienced an exacerbation of their asthma defined as a 0.5 point fall in asthma control questionnaire between screening and symptom day 7 (n=16 exacerbated, n=10 did not). Plots show mean $\pm$ 95% confidence interval.

(B) Plot of areas under the curve (AUC) for peripheral Th17 response over time, stratified by whether subjects experienced an exacerbation. Areas under curves were calculated from the frequency of Th17 cells at each of 8 time-points and then compared using unpaired *t* tests according to the method of Matthews²⁷.

Figure E8. Clinical correlates of mucosal associated invariant T-cells frequencies

Frequencies of V α 7.2+CD161+ (MAIT)-cells in peripheral blood (A,C-F) and sputum (B) are negatively correlated with (A,B) dose of inhaled corticosteroids, (D) asthma control questionnaire score, (E) GINA level of control, (F) step on GINA treatment algorithm and positively correlated with FEV1 (as a percentage of predicted). r_s , Spearman's correlation coefficient.

Figure E9. Additional pathobiologic features of clinico-pathobiologic clusters

Analyses were generated from the clinico-pathobiologic TDA network shown in Figure 4 from the main text. (A) Frequencies of CD3+8- IL-13+ (Th2) cells measured

by flow cytometry in bronchial biopsies are elevated specifically in the mild-moderate clusters A to C. (B) Concentrations of interleukin-13 averaged across serum, sputum and bronchoalveolar lavage. C) and D) Average concentrations of mast cell (C) chymase (ng/ml) and (D) carboxypeptidase A3 in BAL and sputum are markedly elevated in clusters E and F (severe, steroid-refractory disease). Box and whisker plots show medians, interquartile ranges and ranges. Statistical tests: one way ANOVA, with *post-hoc t* tests when compared with health, using Bonferroni correction (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Figure E10. CD4+ T cell subsets in bronchial biopsies according to clinico-pathobiologic clusters

Analyses were generated from the clinico-pathobiologic TDA network shown in Figure 4 from the main text. Frequencies of (A) CD3+8- IL-17+ (Th17) cells, (B) CD3+8-IFN- γ (Th1) cells, (C) CD3+8-IL-13+ (Th2) cells and (D) CD3+8-FoxP3+ (Treg) measured by flow cytometry in bronchial biopsies. Box and whisker plots show medians, interquartile ranges and ranges. Statistical tests: one way ANOVA, with *post-hoc t* tests when compared with health, using Bonferroni correction (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Figure E11. Inflammatory mediators in bronchoalveolar lavage (A,B,D,E) and sputum (C,F) in health and asthma

Concentrations of tryptase (A and D), (B) carboxypeptidase A3 (B and E) in BAL, and sputum of chymase (c and f). Differences are compared by Mann Whitney tests

(two groups). P values are for Kruskal-Wallis tests (multiple groups) with *post hoc* Dunn's compared with health: * $P < 0.05$, ** $P < 0.01$.

Figure E12. Analyses generated from clinico-pathologic TDA network shown in Figure 4.

(A) Asthma severity differs widely between different clinico-pathologic subgroups: named from 1 to 6 in increasing severity using GINA classification of health (0), controlled asthma (1), partially controlled asthma (2) and uncontrolled asthma (3). (B) Dose of inhaled corticosteroids (ICS) in micrograms beclometasone equivalent (mcg bdp) per day. Box and whisker plots show medians, interquartile ranges and ranges. Statistical tests: one way ANOVA, with *post hoc t* tests compared with health, using Bonferroni correction (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Figure E13. Other features amongst clinico-pathobiologic clusters

(A) Clinico-pathobiologic network colored according to average IL-13-secreting Th2 cell frequencies in blood, sputum, BAL and biopsies (red, high frequencies; blue, low frequencies). (B) Distribution of asthmatics fulfilling the criteria of 'cohort 5' defined by the Severe Asthma Research Program.⁸ (C) Aspirin-sensitive asthma (red: present, blue: absent, green / yellow: mixed nodes containing some subjects with and some without aspirin-sensitivity). (D) Frequencies of IL-13 secreting CD8+ (Tc2) cells in bronchial biopsies; note the close correlation, i.e. co-localization, with sputum eosinophilia.

The TDA used 62 subjects with most complete data; Metric: variance normalized Euclidean; Lenses: principal and secondary singular value decomposition (SVD)(Resolution 32, Gain 4.0/3.5x, Equalized); Node size: proportional to number of individuals in node. Color bars: red high values, blue: low values.

Figure E14. Peripheral blood MAIT-cell frequencies over the course of the year and after oral corticosteroids

(A and B) Frequencies of peripheral blood MAIT (V α 7.2+CD161+) cells and non-MAIT T-cells (V α 7.2+CD161- cells) in 12 moderate asthmatic subjects, usually controlled on inhaled corticosteroids, before and after 7 days of treatment with 20 milligrams of oral prednisolone given once daily. Frequencies are shown as a percentage of live CD3+ T-cells. P values are for paired *t* tests: * P<0.05, ** P<0.01.

(C) Peripheral blood MAIT-cell frequencies and (d) serum vitamin D3 concentrations vary over the course of the year and are highest in the summer months. (c) Log-transformed V α 7.2+CD161+ cell frequencies from healthy and asthmatic subjects according to the quarter in which phlebotomy was performed, with a sinusoidal regression line. $R^2=0.16$. (D) Log-transformed vitamin D3 concentrations from healthy and asthmatic subjects according to the quarter in which phlebotomy was performed, with a sinusoidal regression line. $R^2=0.30$.

582 **Tables**

583

584 **Table E1. Criteria used to attribute physician assessed severity of asthma at**
 585 **the time of enrolment**

586

Mild asthma
Symptoms < once a day Nocturnal symptoms < twice a month • FEV₁ or PEF ≥80% predicted Treatment: • Salbutamol as needed only Presence of asthma confirmed by methacholine challenge testing.
Moderate asthma
Symptoms < once a day Nocturnal symptoms <once a week Asthma control score >1.5 Treatment: • Salbutamol as needed only • Low-dose inhaled steroids (<800 mcg beclometasone dipropionate equivalent) • +/- Long acting beta-2-agonist

Presence of asthma confirmed by methacholine challenge testing.

Severe asthma

Symptoms daily

Nocturnal symptoms >once a week

Daily use of inhaled short-acting β_2 -agonist

- FEV₁ or PEF <80% of predicted or patient's best

Treatment:

- High-dose inhaled steroids (≥ 800 mcg beclometasone dipropionate equivalent)
- Long acting beta-2-agonist
- +/- frequent or continuous oral corticosteroids

587 These criteria² were agreed before the study began and used by T.S.C.H. to assign
588 each participant with asthma to one of 3 categories of disease severity after full
589 clinical and physiologic assessment during screening/enrolment. Where patients did
590 not map on to a single category they were considered on an individual basis to
591 achieve the best possible fit.

592 **Table E2. Definition of terms used in the topological data analysis**

Term	Definition	Compartments	Assay
T-cell subsets			
Th1	CD3+CD4+IFN γ + cells	Composite	9 color
Th2	CD3+CD4+IL-13+ cells	average across blood, sputum,	fluorescence activated flow
Th17	CD3+CD4+IL-17+ cells	BAL, bronchial	cytometry
Treg	CD3+CD4+FOXP3+ cells	biopsy	
Tc1	CD3+CD8+IFN γ + cells		
Tc2	CD3+CD8+IL-13+ cells		
MAIT	CD3+V α 7.2+CD161+ (MAIT) cells		
Cytokines			
IFN- γ	Interferon- γ concentrations	Composite	Electrochemi-
IL-2	Interleukin-2 concentrations	average across blood, sputum,	luminescent assay
IL-4	Interleukin-4 concentrations	BAL	
IL-5	Interleukin-5 concentrations		
IL-10	Interleukin-10 concentrations		
IL-12p70	Interleukin-12p70 concentrations		

IL-13	Interleukin-13 concentrations		
Mast cell mediators			
Chymase	Chymase concentrations	Composite	ELISA
Carboxypeptidase A3	Carboxypeptidase A3 concentrations	average across sputum, BAL	
Tryptase	Tryptase concentrations		
Inflammatory cells			
Neutrophils	Neutrophil frequencies	Composite	Cell counts
Lymphocytes	Lymphocyte frequencies	average across sputum, BAL	
Macrophages	Macrophage frequencies		
Eosinophils	Eosinophil frequencies	Composite	
		average across blood, sputum, BAL	
Inflammatory subtype			
Neutrophilic	Sputum subclass: neutrophils >61% ²⁸	Sputum	Cell counts
Eosinophilic	Sputum subclass: sputum eosinophils >3% ²⁹	Sputum	

Paucigranulocytic	Sputum subclass: neutrophils $\leq 61\%$, ²⁸ sputum eosinophils $\leq 3\%$	Sputum	
Other			
eNO	Exhaled nitric oxide levels (ppb)	Exhaled breath	Niox Mino® at 50mL/s
IgE	Serum immunoglobulin E	Serum	ELISA
Vitamin D3	Vitamin D3 concentration	Serum	Mass spectrometry
Clinical			
BMI	Body Mass Index		
FEV ₁	Spirometric forced expiratory volume in 1 second (L), pre- bronchodilator		
Reversibility	Percentage change in FEV ₁ before and 15 minutes after 500 micrograms nebulized salbutamol		
GINA severity	Asthma severity according to GINA classification ⁹ assigned values:(0) health, (1) controlled asthma, (2) partially controlled		

asthma, (3) uncontrolled
asthma.

Physician Asthma severity assessed by a
assessed severity physician (T.S.C.H.) using
criteria in Table E1

593

594

595 **Table E3. Parameters included in the topological data analyses**

596

Pathologic parameters ^A							
eNO	IgE						
IL-4	IL-5	IL-13	IL-17	IL-2	IL-12p70	IFN-γ	IL-10
Th17	Th1	Th2	Treg	Tc1	Tc2	MAIT	
Macrophages		Lymphocytes		Neutrophils		Eosinophils	
Tryptase	Chymase	Carboxypeptidase A3		Basogranulin		Vitamin D3	
Clinical parameters							
Age	Gender	Inflammatory subtype		ACQ	BMI		
Age of onset of asthma				Atopic status			
History of symptoms induced by foods				History of smoking (pack years)			
History of virus-induced exacerbations				History of eczema			
History of exercise-induced symptoms				History of allergic rhinitis			
History of symptoms induced by aerosols				History of nasal polyposis			
History of symptoms induced by emotion							
History of symptoms induced by aeroallergens							
History of symptoms induced by changes in air temperature of humidity							

History of symptoms induced by salicylates ^B

Inhaled corticosteroid dose

Oral corticosteroid dose

Use of long acting beta-2 agonists

Use of leukotriene receptor antagonists

Peak expiratory flow (percent predicted)

FEV₁ (pre-bronchodilator)

Bronchodilator reversibility

597

598 ^AOther parameters which incorporate *a priori* assumptions or overlap extensively
599 other parameters were not used to generate the main TDA network: physician
600 assessment of severity, GINA severity, treatment burden (step on GINA treatment
601 algorithm). ^B 'Salicylate' refers to symptoms induced by either salicylates or non-
602 salicylate non-steroidal anti-inflammatory drugs.

604 **Table E4. Descriptions of clinico-pathobiologic clusters**

Endo- type	Features of cluster ^A	Kolmogorov- Smirnov tests		Comments
		KS	P	
		score	value	
1	Clinical			
	Lower asthma severity by enrolment criteria	0.643	0.000 5	Mostly physician assessed as 'mild'
	Lower GINA severity	0.482	0.02	Mostly GINA severity 'controlled'
	Lower ACQ	0.434	0.04	Mean ACQ 0.88
	Lower treatment step (GINA)	0.699	0.000 1	Mostly GINA treatment step 1
	Lower ICS Dose	0.607	0.001	Mostly not on ICS
	Higher FEV ₁ (% pred)	0.496	0.01	Mostly atopic
	Higher PEFR (% pred)	0.496	0.01	
	More likely paucigranulocytic sputum	0.430	0.047	
	Pathobiologic			

	Higher Th2	0.485	0.02	
	Lower tryptase	0.585	0.002	
	Lower IL-13	0.430	0.047	
	Lower vitamin D3	0.515	0.01	
2	Clinical			
	Lower ACQ	0.690	0.02	Mean ACQ 0.5
	Lower BMI	0.667	0.02	
3	Pathobiologic			
	Higher Th17	0.607	0.02	Mostly physician assessed as 'moderate'
	Higher Th1	0.585	0.03	Mostly GINA severity 'partially controlled'
	Higher IL-5	0.561	0.04	Highest reversibility (mean 18%)
	Higher IL-12p70	0.607	0.02	Highest eNO (mean 65 ppb)
	Higher IFN-γ	0.604	0.025	
4	Pathobiologic			Mostly physician assessed as 'moderate'
	Lower IL-17	0.861	0.02	Highest age of onset (median 33 years)
				Highest frequency of nasal polyps and salicylate sensitivity ^B

5	Clinical			
	Older age	0.556	0.03	Mean age 50 years
	Lower PEFr (% pred)	0.530	0.04	High BMI (mean 32.6)
	Higher treatment step (GINA)	0.581	0.02	Mostly GINA treatment step 4/5
	Higher ICS dose	0.598	0.01	Mean ICS dose 1500 mcg
	Pathobiologic			High ACQ (mean 2.1)
	Higher tryptase	0.667	0.004	
	Higher IL-13	0.513	0.049	
	Higher Tc2	0.539	0.03	
	Lower basogranulin	0.530	0.038	
6	Clinical			
	Higher ACQ	0.744	0.003	Highest ACQ (mean 3.2)
	Higher treatment step (GINA)	0.670	0.009	Mostly GINA treatment step 5
	Higher ICS dose	0.693	0.006	Mean ICS dose 1400 mcg
	Higher LTRA use	0.721	0.004	Highest BMI (mean 35.3)
	Higher use of reliever medication	0.663	0.02	Mostly female (88%)

Higher age of onset	0.556	0.047	High median age of onset 30
Lower FEV ₁ (% pred)	0.647	0.01	Lowest FEV ₁ (mean 62% pred)
Pathobiologic			High frequency of salicylate sensitivity ^B
Higher tryptase	0.619	0.02	50% of group OCS (mean 14mg
Higher carboxypeptidase A3	0.571	0.04	prednisolone/day)
Lower MAIT	0.593	0.03	
Lower Th2	0.564	0.04	
Lower Th1	0.622	0.02	
Lower Th17	0.593	0.03	
Lower Tc1	0.644	0.014	

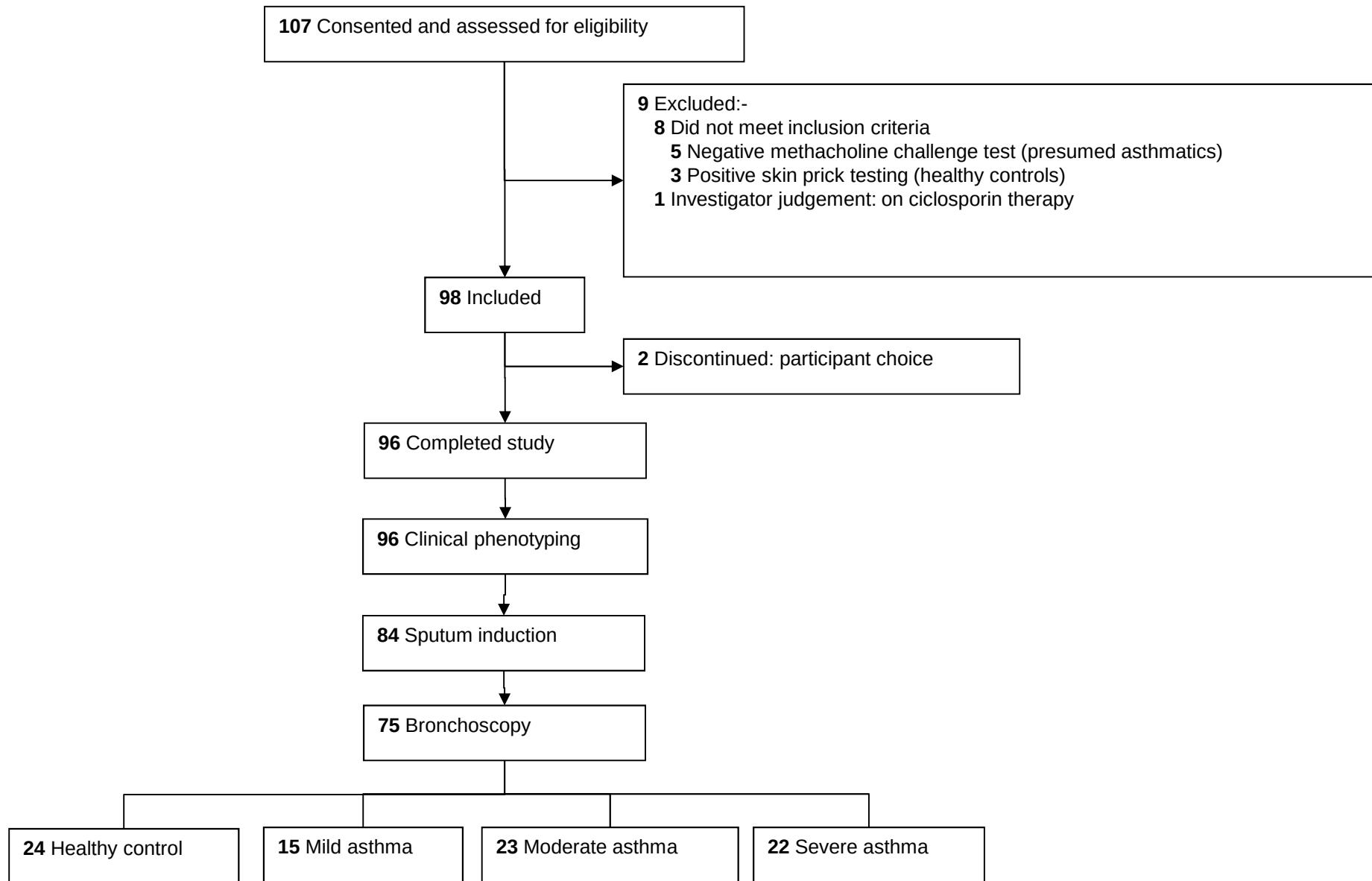
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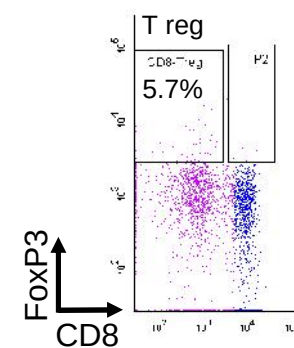
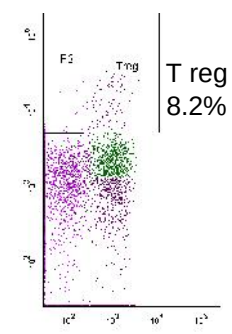
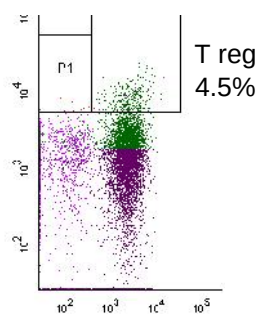
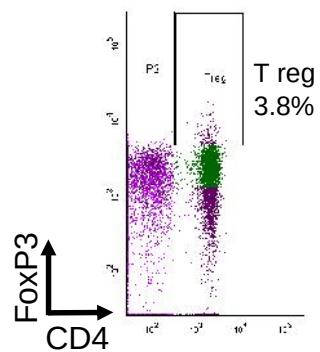
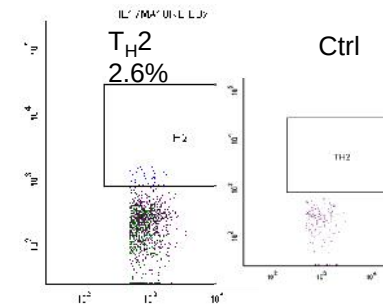
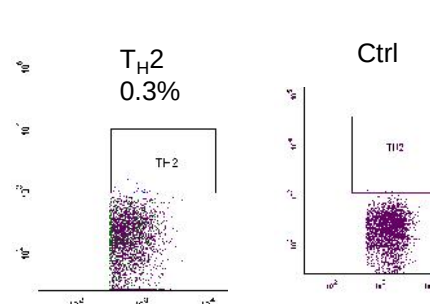
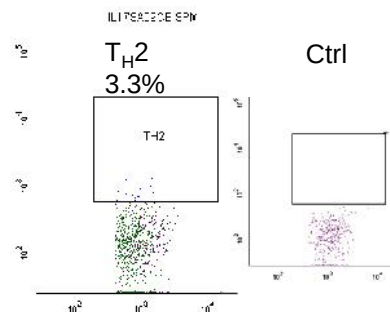
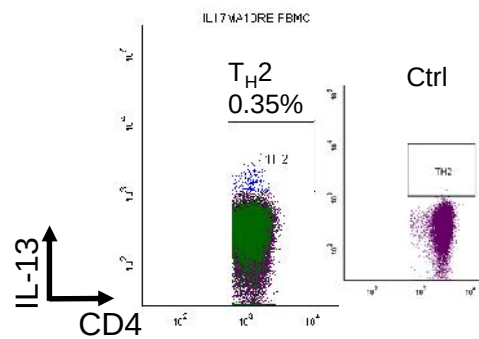
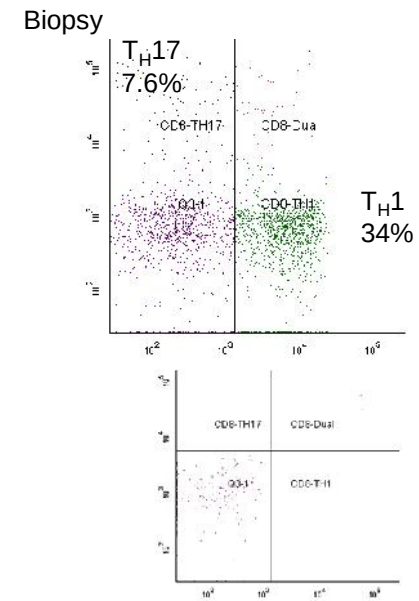
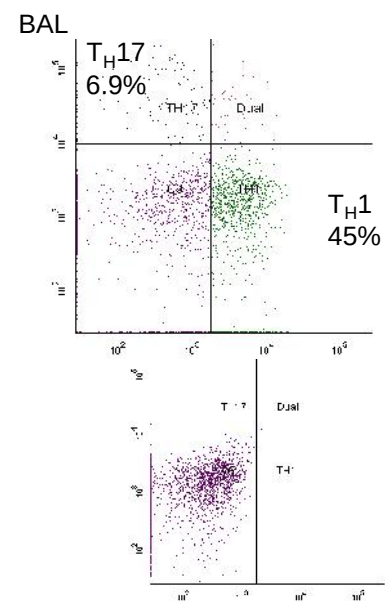
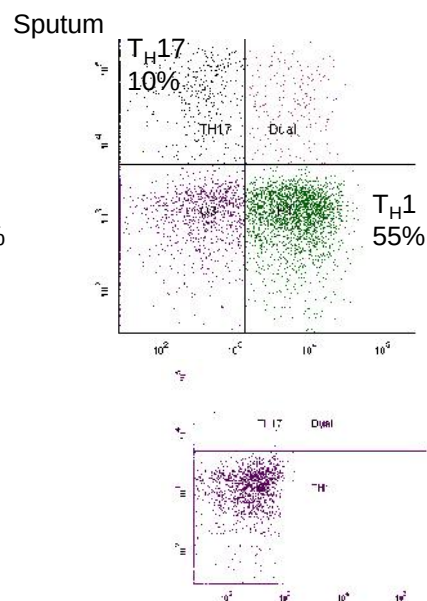
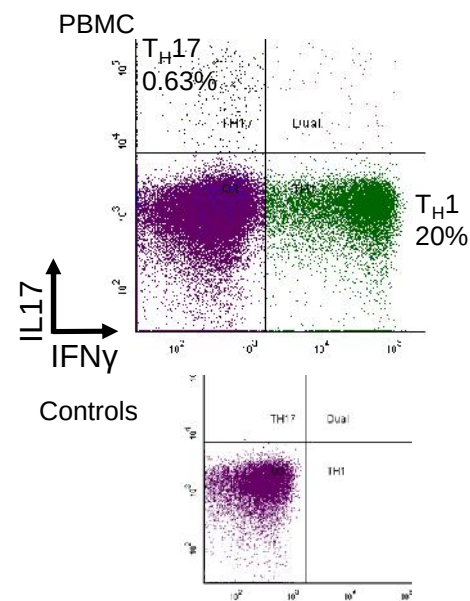
606 ^A Features of each cluster are parameters that differ significantly in Kolmogorov-
607 Smirnov tests between each cluster and a group comprised of the remaining
608 participants. ^B 'Salicylate' refers to symptoms induced by either salicylates or non-
609 salicylate non-steroidal anti-inflammatory drugs. Abbreviations: ACQ, Seven point
610 Asthma Control Score;¹⁰ BMI, body mass index; GINA, Global Initiative for Asthma;⁹
611 ICS, inhaled corticosteroids; OCS, oral corticosteroids. For other terms see table E2.

References

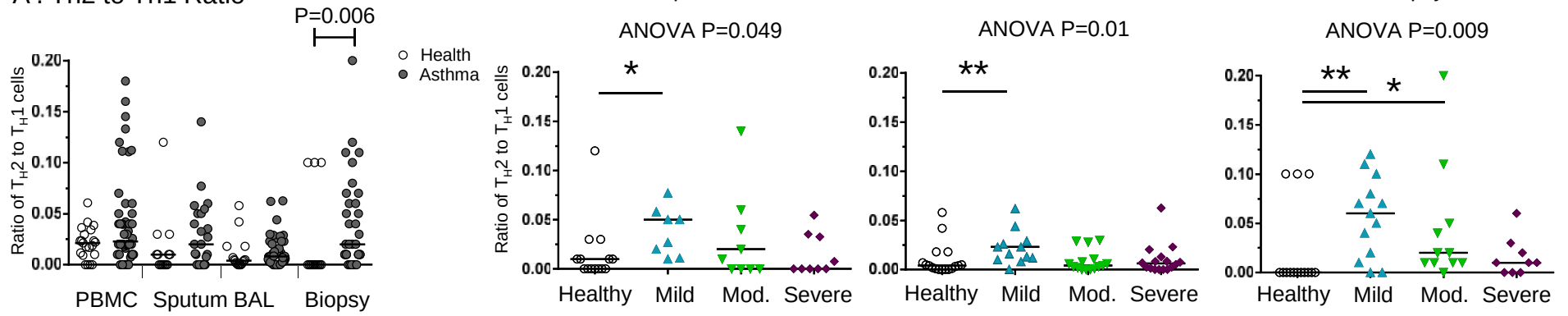
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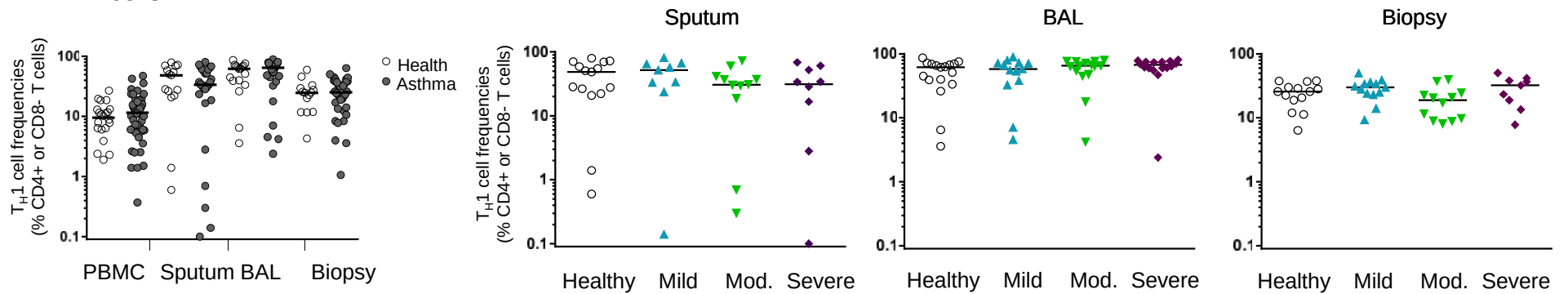




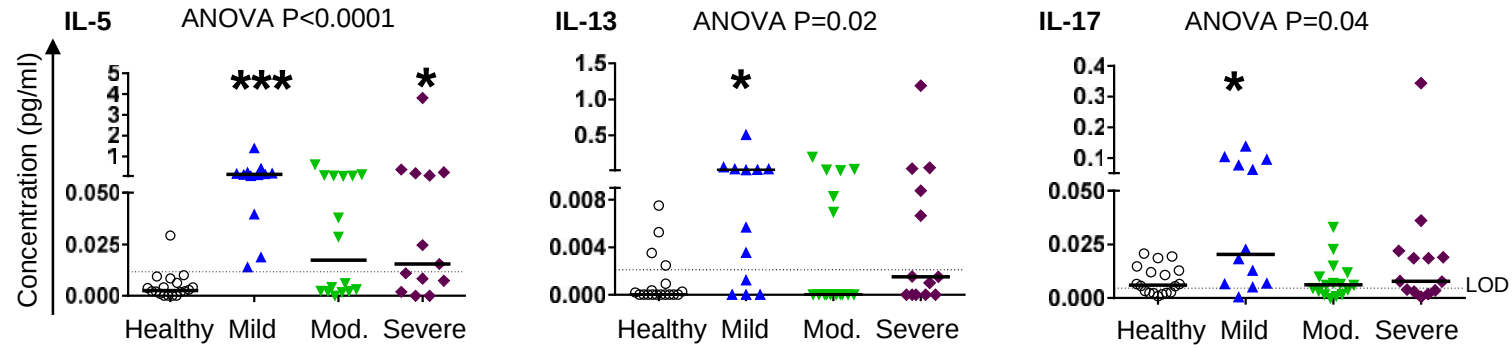
A. Th2 to Th1 Ratio



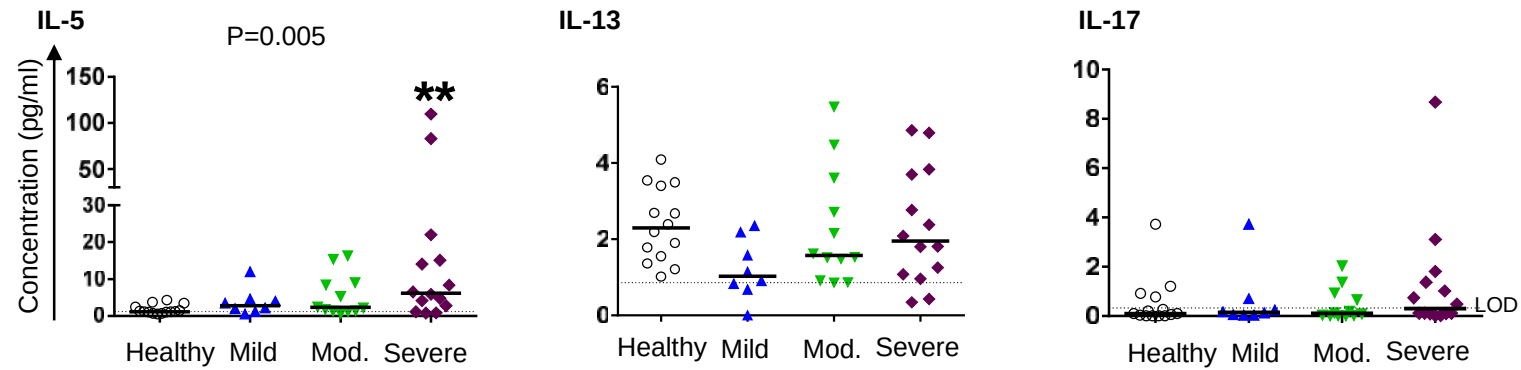
B. Th1 cells



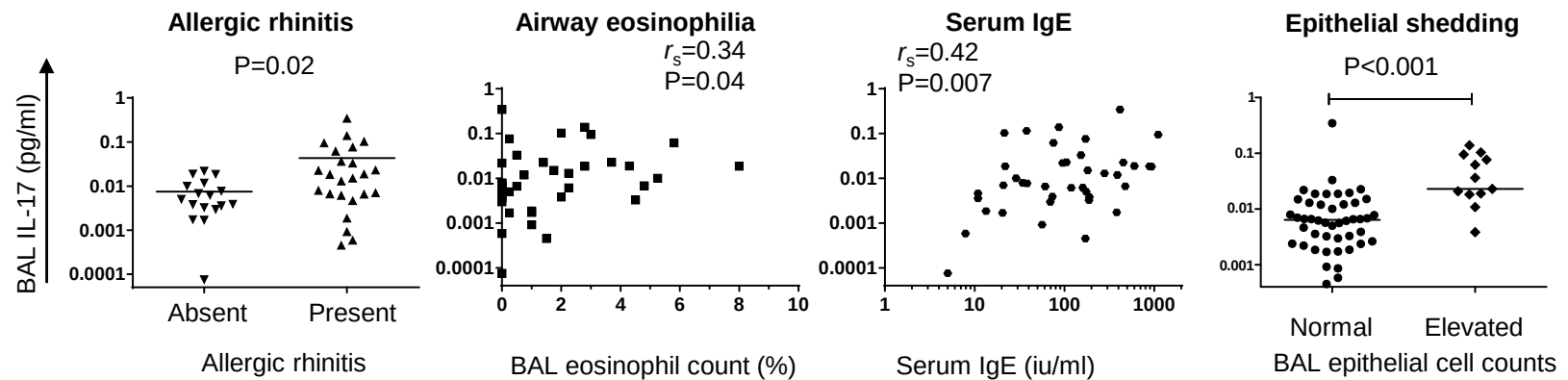
A. BAL cytokines



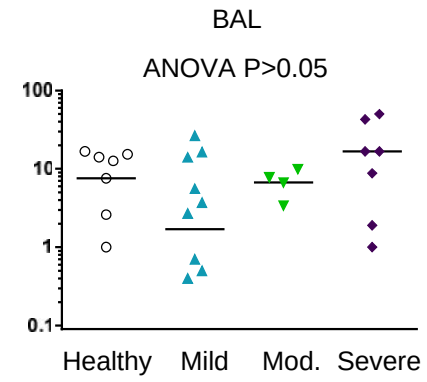
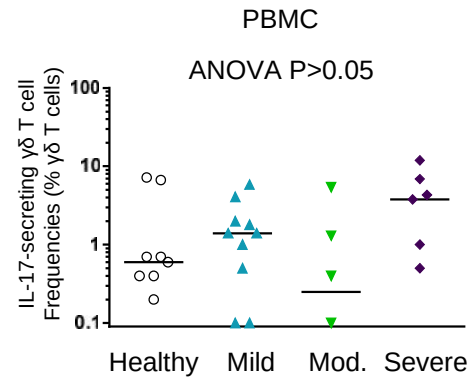
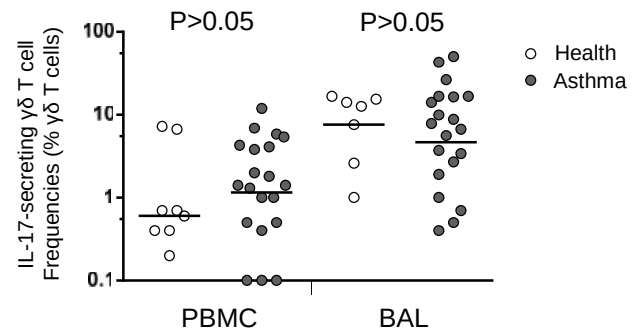
B. Sputum Supernatant

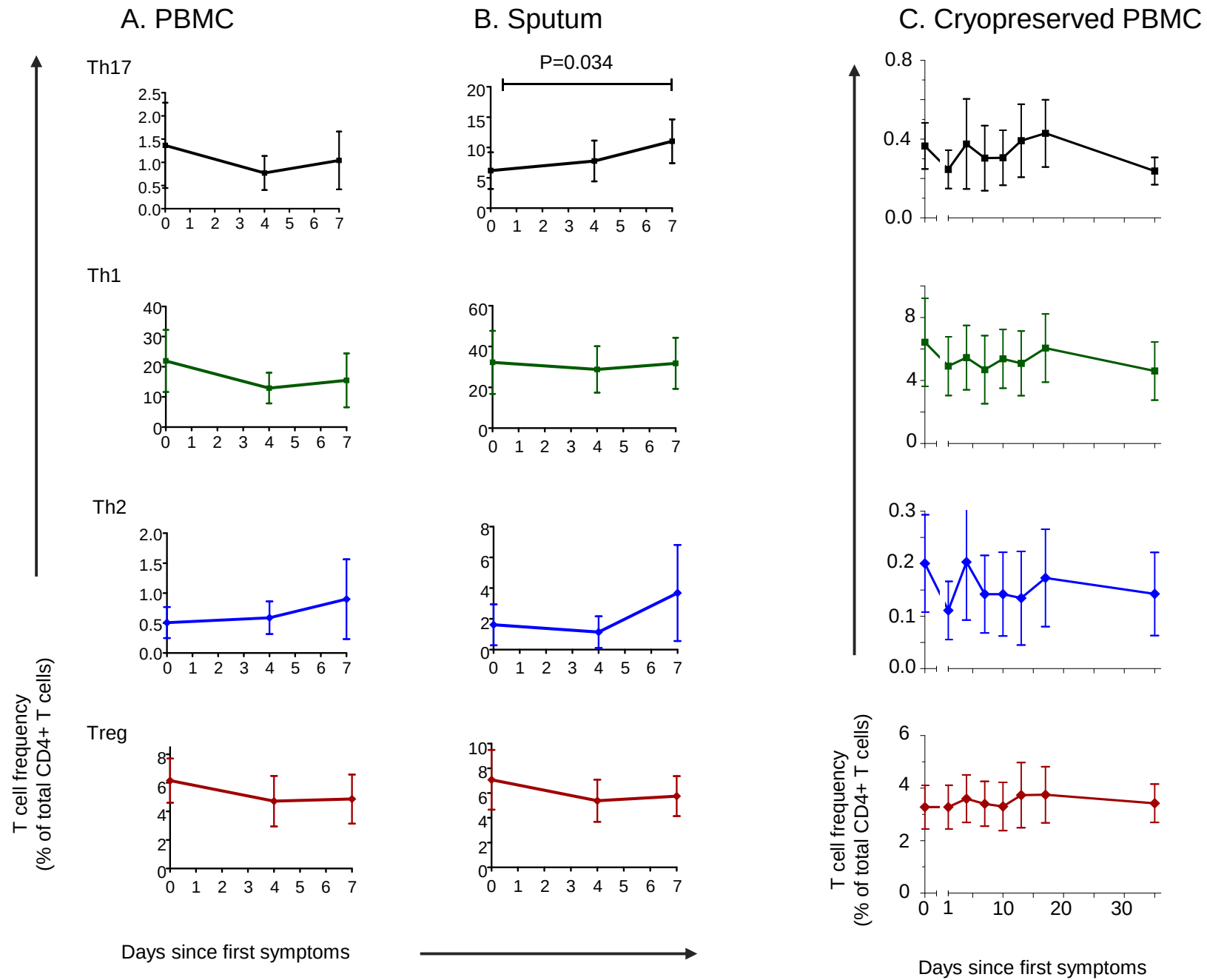


C. Clinical Correlates of BAL IL-17

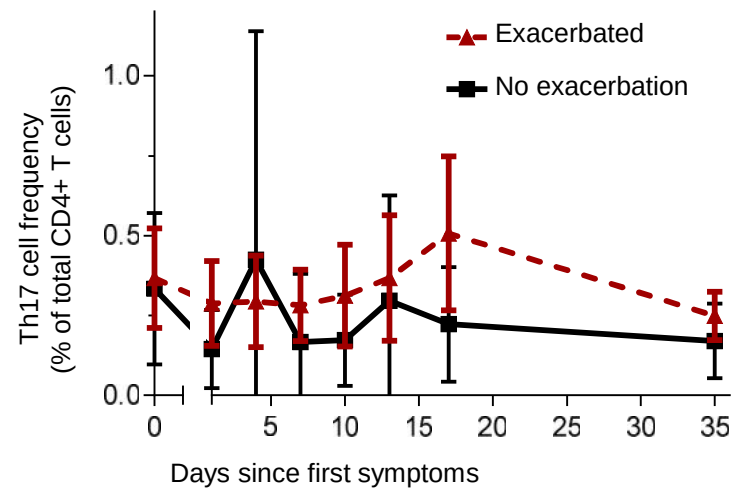


$\gamma\delta$ -17 T cells

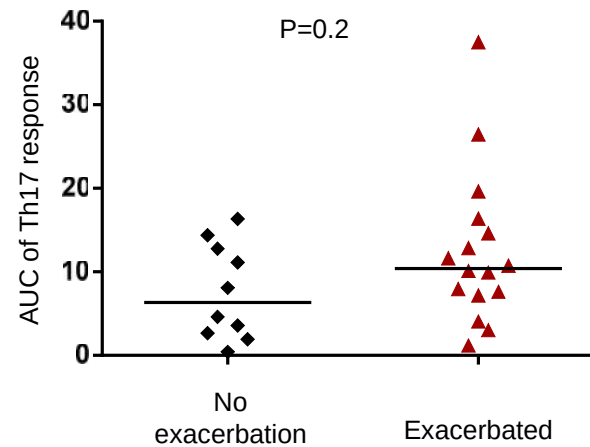




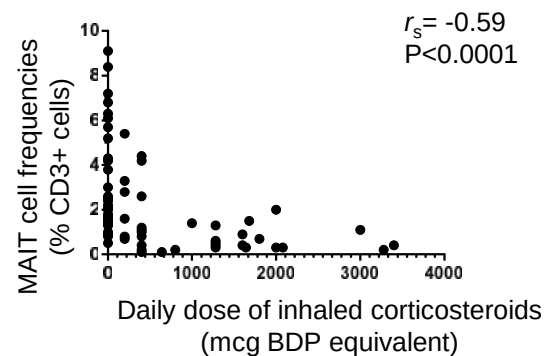
A. Dynamics of peripheral Th17 cell frequencies



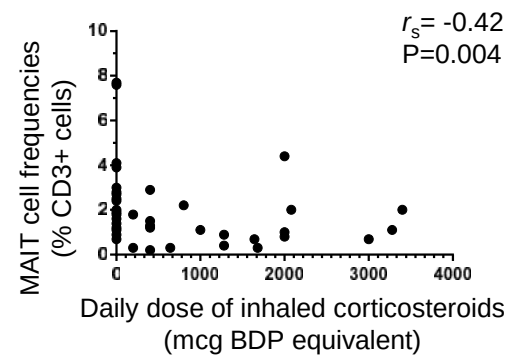
B. Areas under curves for Th17 response



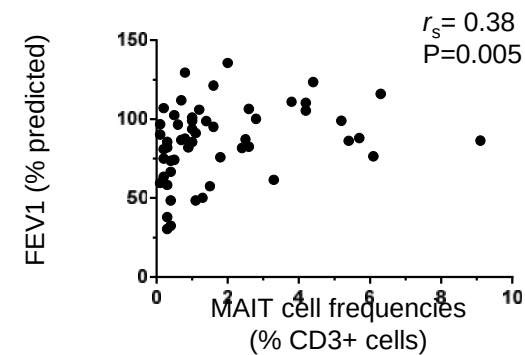
A. PBMC MAIT cells v ICS dose



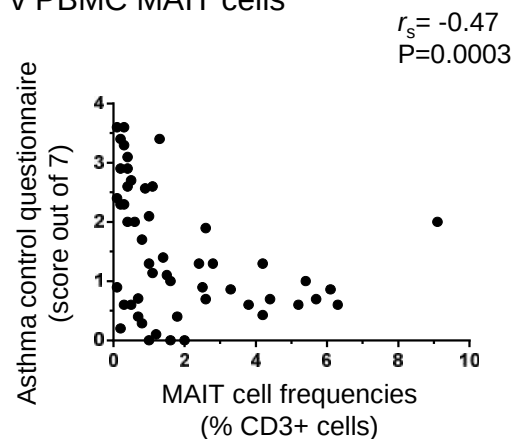
B. Sputum MAIT cells v ICS dose



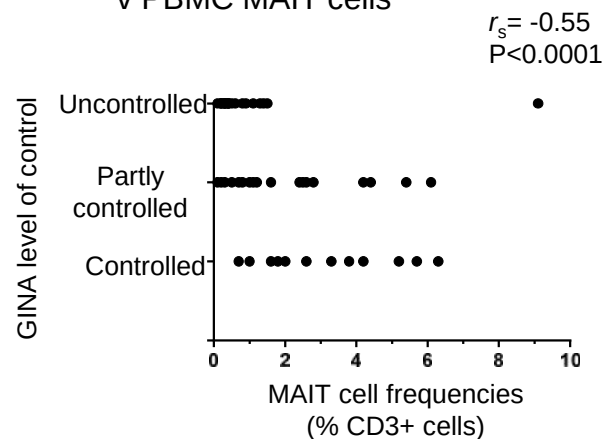
C. FEV1 v PBMC MAIT cells



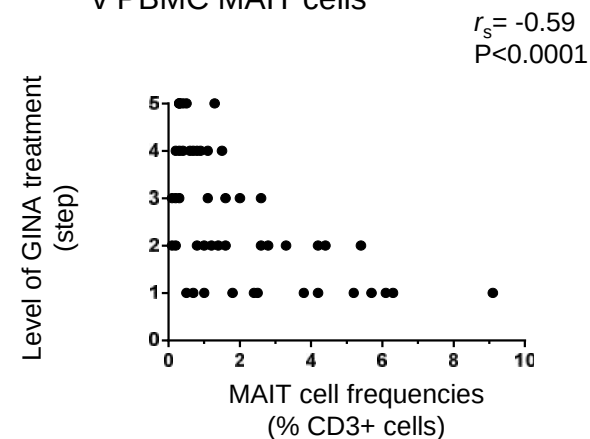
D. Asthma control questionnaire v PBMC MAIT cells



E. GINA level of control v PBMC MAIT cells

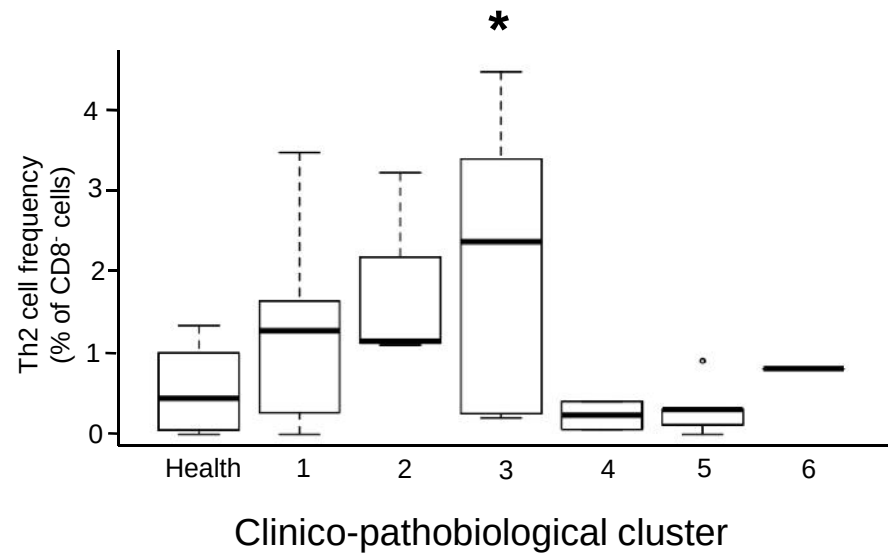


F. Step on GINA treatment algorithm v PBMC MAIT cells



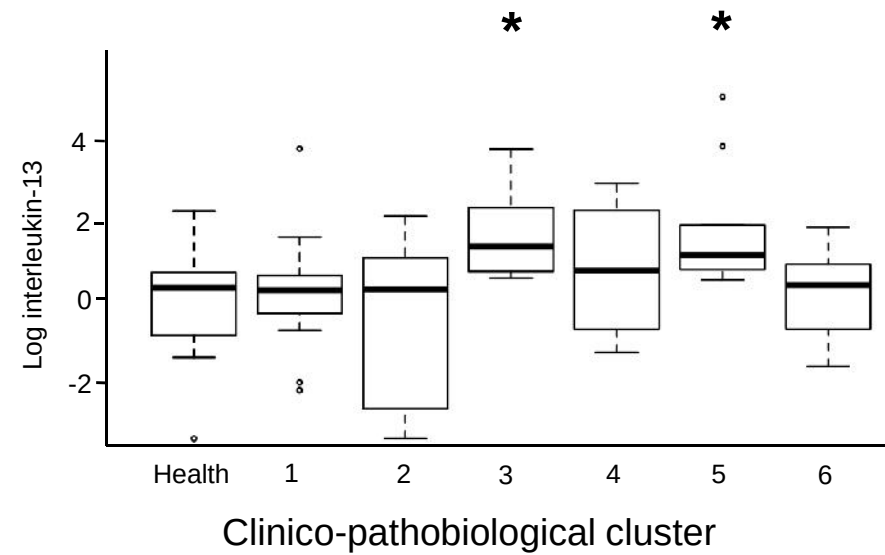
A. Th2 cell frequencies in bronchial biopsies

ANOVA P=0.02



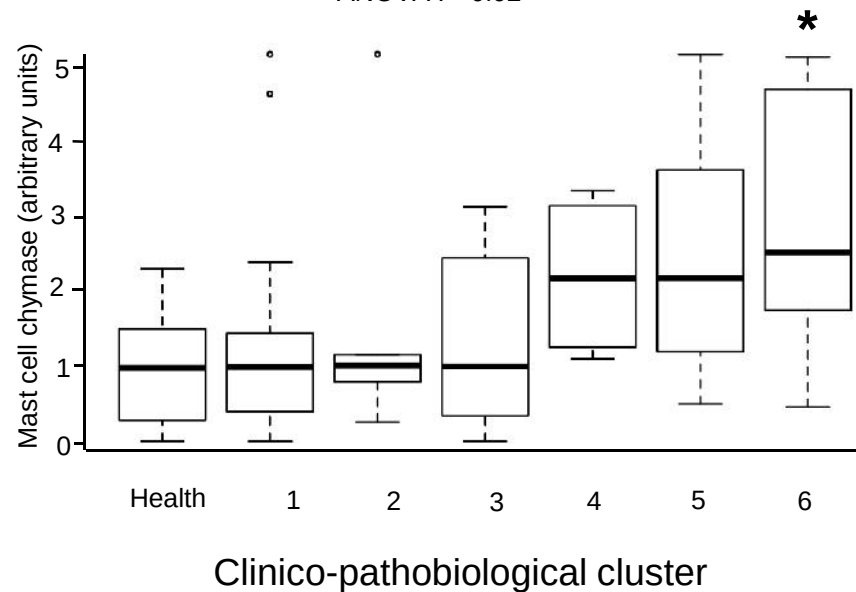
B. Interleukin-13

ANOVA P=0.02



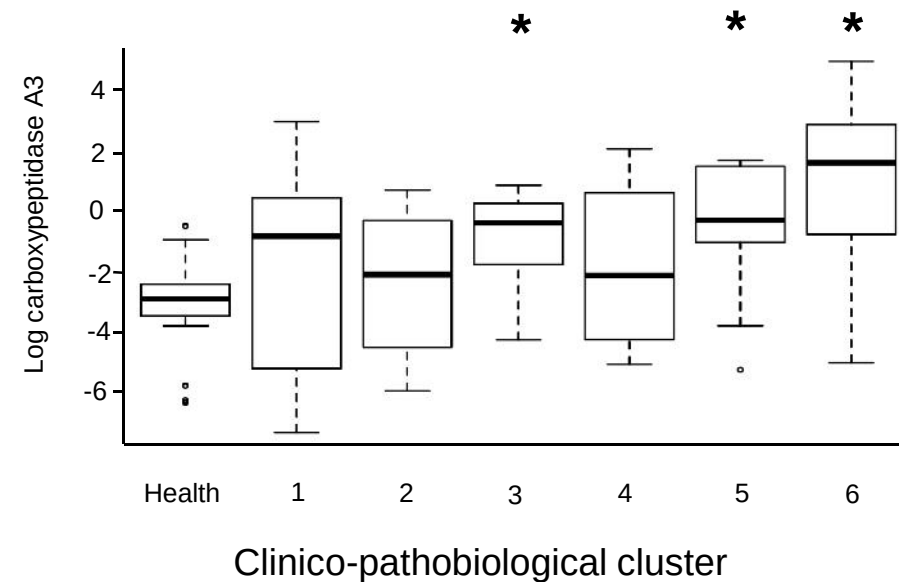
C. Mast cell chymase

ANOVA P=0.02



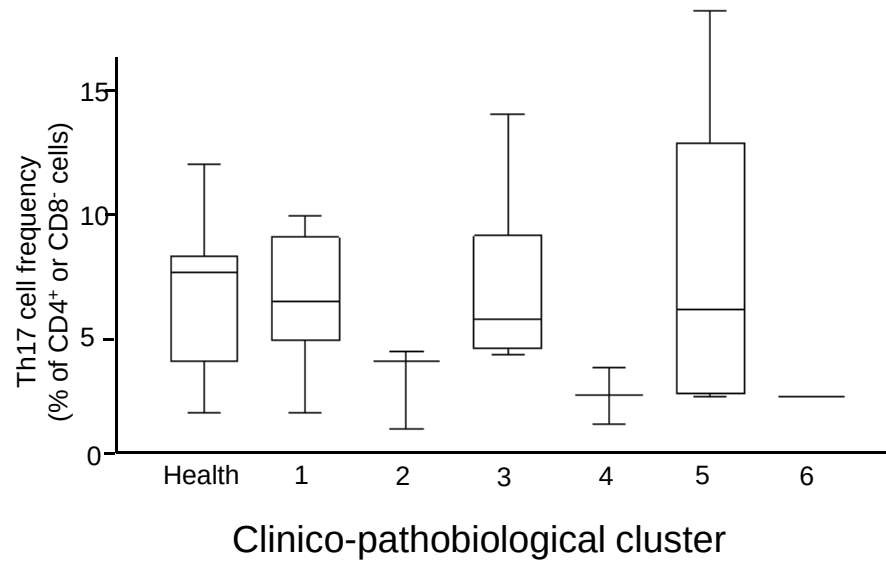
D. Carboxypeptidase A3

ANOVA P=0.02



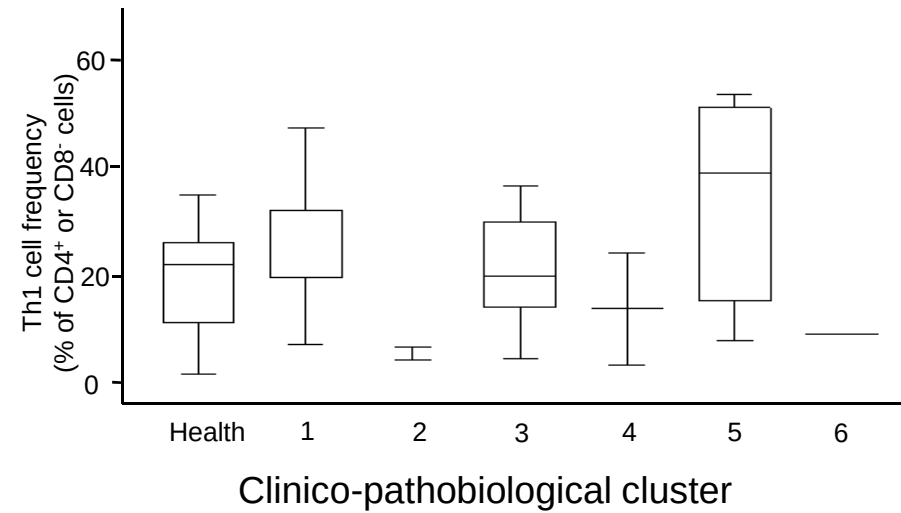
A. Th17 cell frequencies in biopsies

ANOVA P=0.3



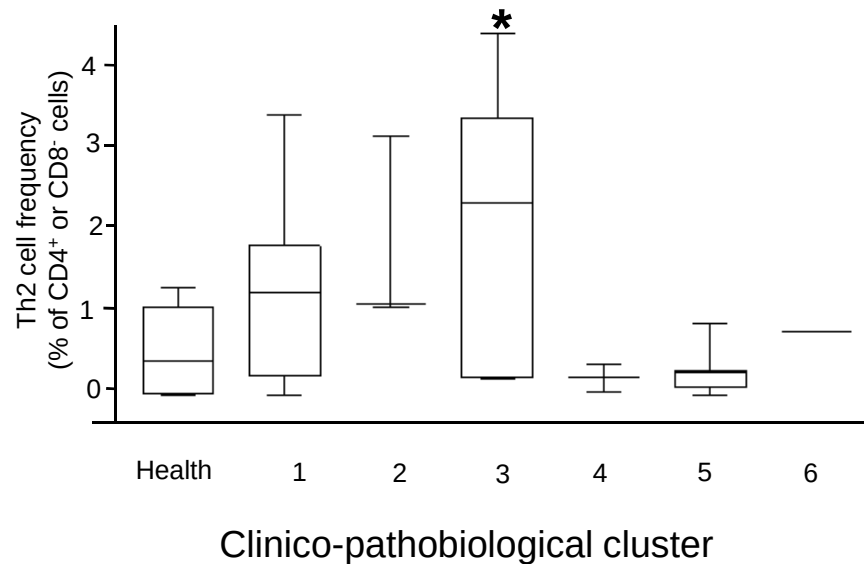
B. Th1 cell frequencies in biopsies

ANOVA P=0.02



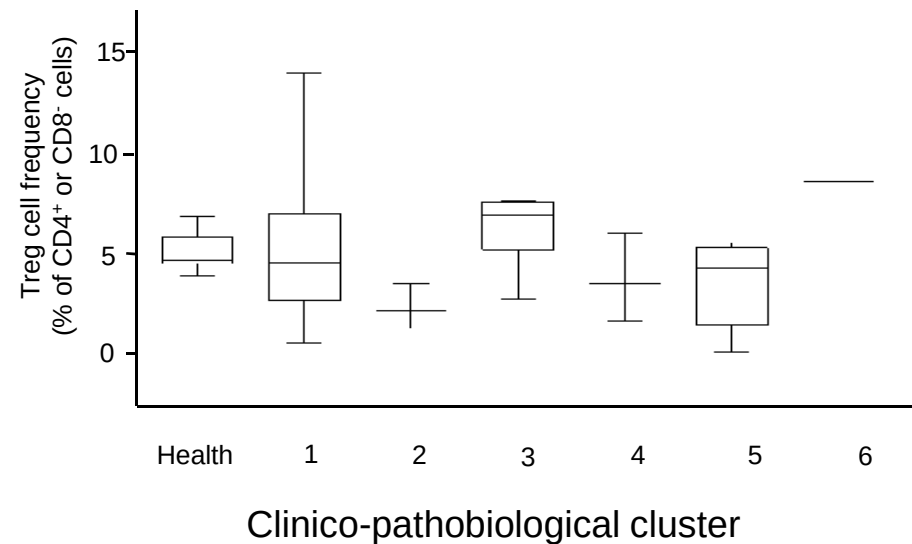
C. Th2 cell frequencies in biopsies

ANOVA P=0.02



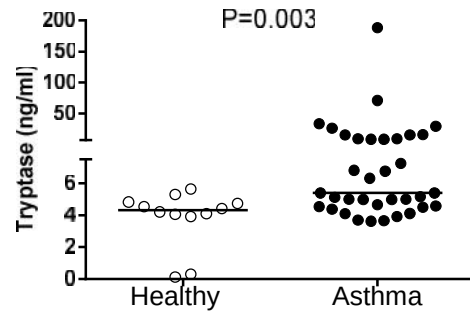
D. Treg frequencies in biopsies

ANOVA P=0.1

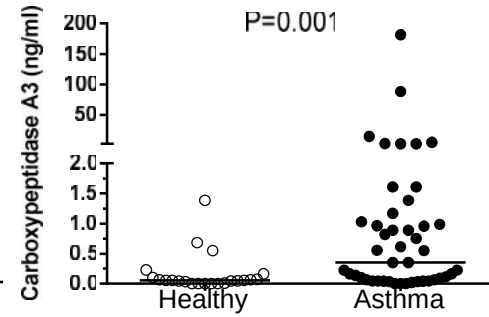


Inflammatory mediators in BAL (a, b, d, e) and sputum (c, f) in health and asthma

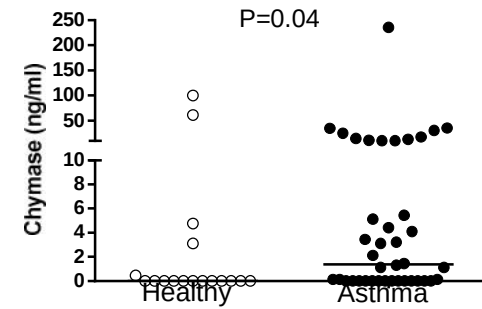
A



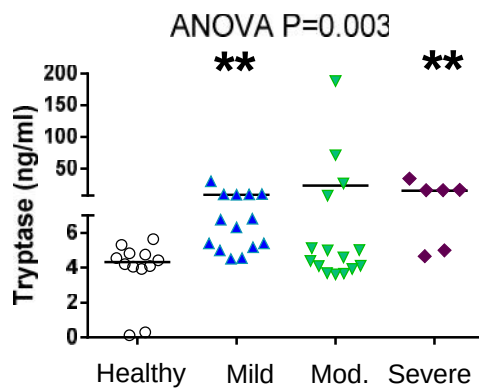
B



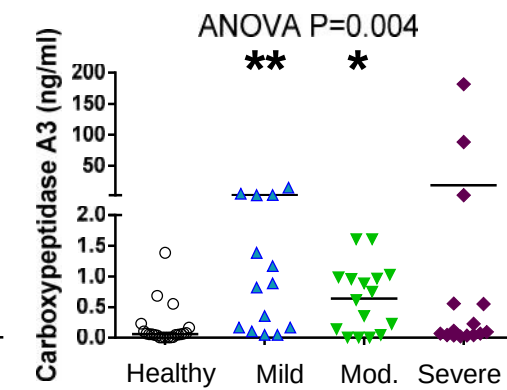
C. Sputum



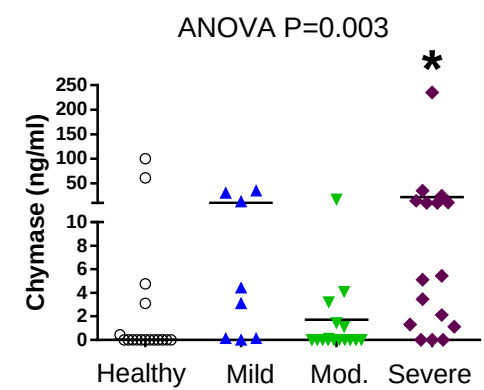
D



E

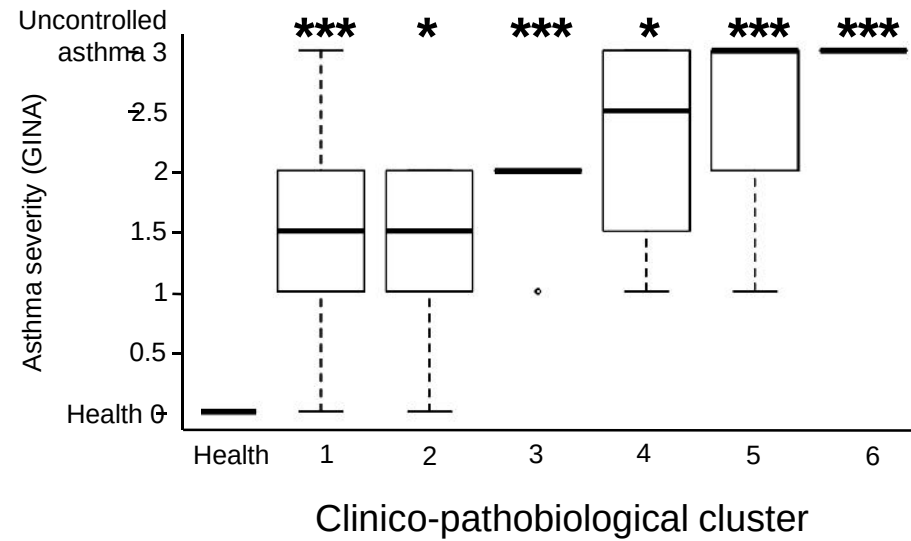


F. Sputum



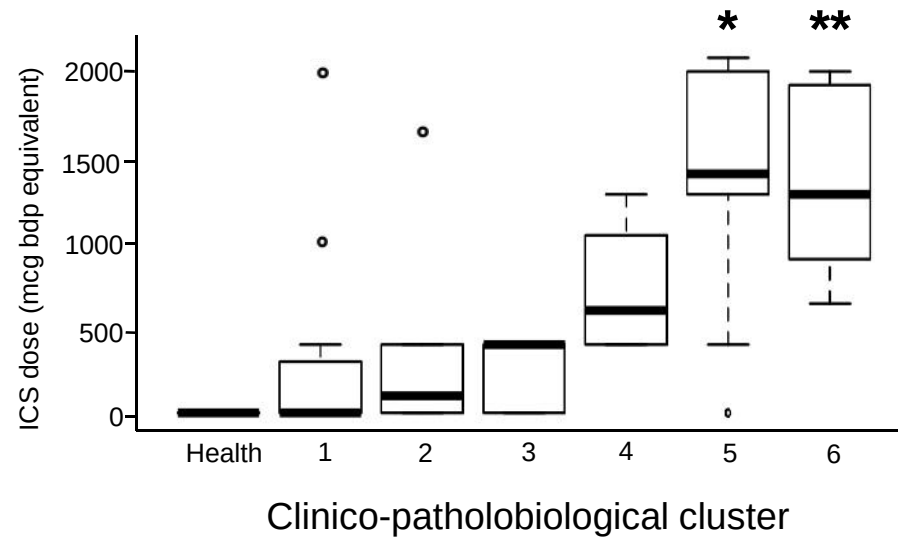
A. Asthma severity

ANOVA $P < 0.001$

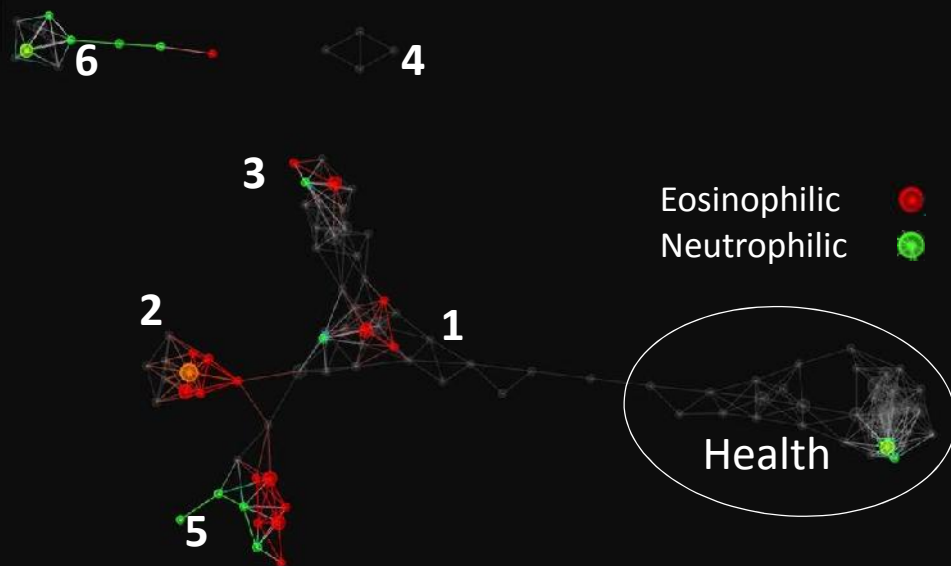


B. Inhaled corticosteroid dose

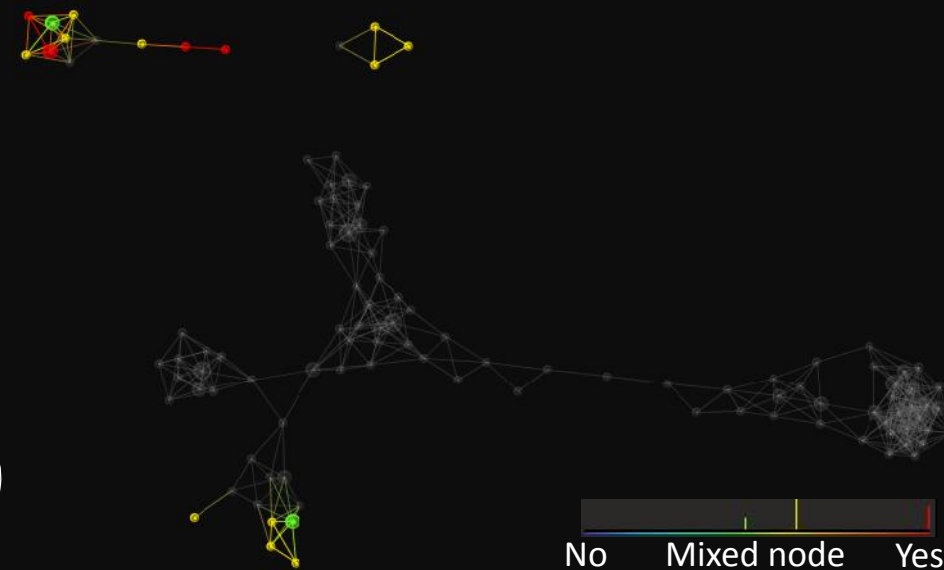
ANOVA $P < 0.001$



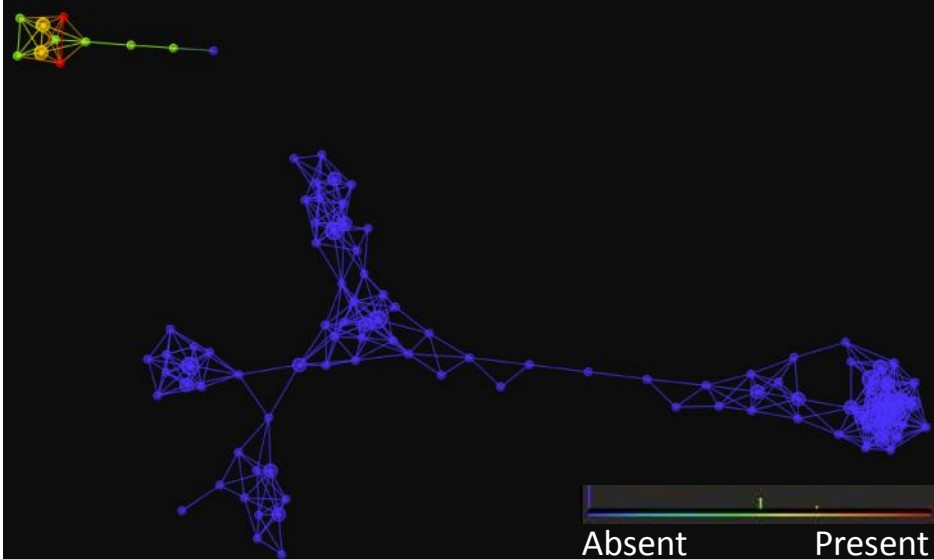
A. Sputum eosinophilia and neutrophilia



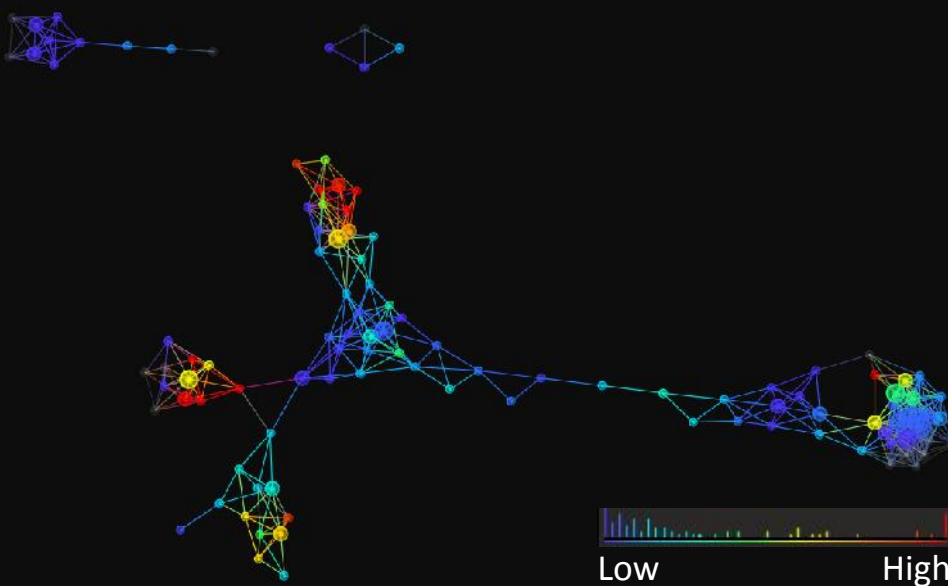
B. SARP: 'cluster 5'



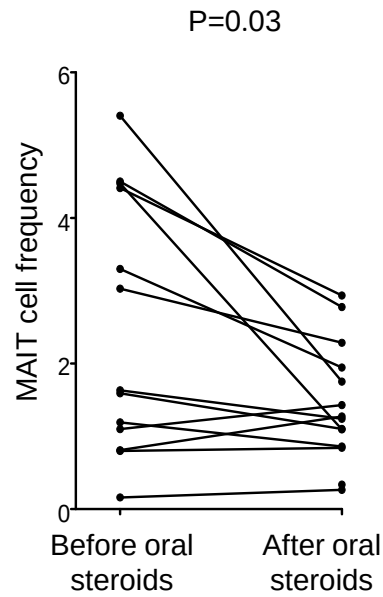
C. Salicylate sensitivity



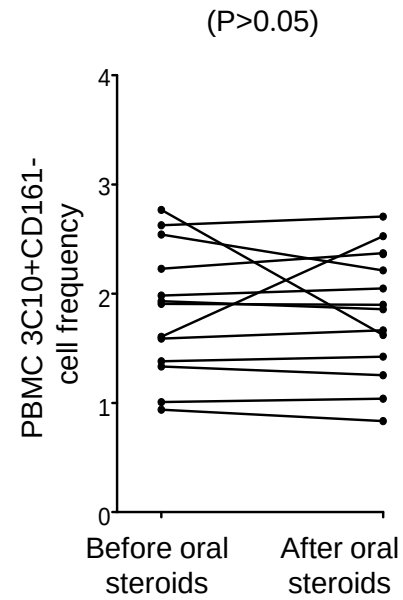
D. IL-13 secreting CD8+ (Tc2) cells in biopsies



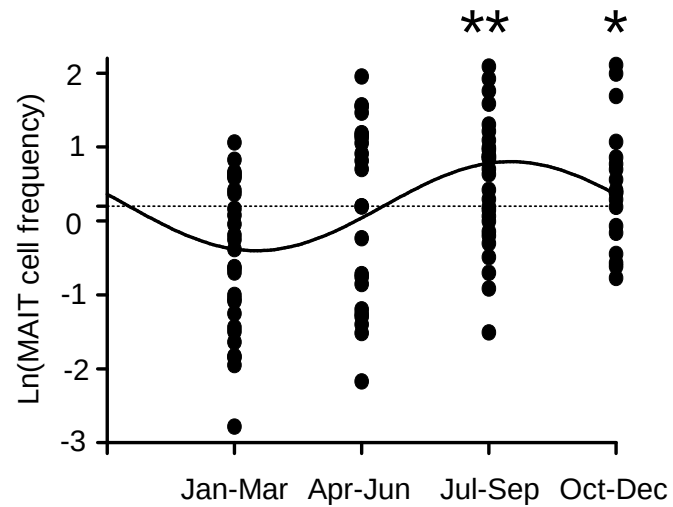
A. MAIT cells



B. Non-MAIT T cells



C. Peripheral MAIT cells by date of sample acquisition (quarter).



D. Serum Vitamin D3 by date of sample acquisition (quarter).

