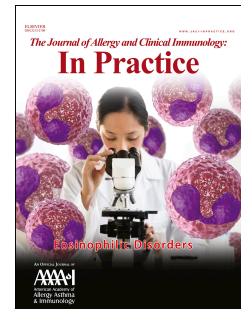


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The prevalence of subtypes of type-2 inflammation in an unselected population of patients with severe asthma.

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Title:

The prevalence of subtypes of type-2 inflammation in an unselected population of patients with severe asthma.

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Conflicts of interest:

The authors have no conflicts of interest to declare.

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Highlights:

What is already known?

Severe T2 high asthma is recognised as a heterogeneous entity and is considered to include several different clinical phenotypes of asthma; however, the prevalence of isolated- and concomitant biomarker elevation is unknown.

What does this article add to our knowledge?

We found 70% of patients with severe asthma to have at least one T2 biomarker elevated: 31% with a single biomarker elevated and 39% with two or more elevated.

How does this study Impact current management guidelines?

Our findings illustrate the heterogeneity of T2 inflammation in severe asthma, supports the existence of distinct subtypes of T2 high asthma and point to a large discrepancy between airway- and systemic eosinophilia.

Keywords:

- T2 inflammation
- Severe asthma
- Phenotypes
- Biomarkers
- Heterogeneity
- Targeted therapy

Abbreviations:

ACQ-5: 5-item asthma control questionnaire

ATS: American Thoracic Society

AUC: Area under the curve

B-EOS: Absolute blood eosinophil count

CDC: Cell differential count

COPD: Chronic Obstructive Pulmonary Disease

DREAM: Mepolizumab for Severe eosinophilic asthma

ERS: European Respiratory Society

FeNO: Fractional Exhaled Nitric Oxide

FEV₁: Forced expiratory volume in 1 second

ICS: Inhaled corticosteroids

IgE: Immunoglobulin E

ILC2: Group 2 innate lymphoid cells

IL-4: Interleukin 4

IL-5: Interleukin 5

IL-13: Interleukin 13

IL-4R α : Interleukin 4 receptor alfa

MENSA: Mepolizumab as Adjunctive Therapy in Patients with Severe Asthma

OCS: Oral corticosteroids

SARP: Severe Asthma Research Programme

SD: Standard deviation

S-EOS: Sputum eosinophil count in percent predicted

T2: Type 2 inflammation

U-BIOPRED: Unbiased Biomarkers in Prediction of respiratory disease outcomes

QUEST: A Randomised, Double Blind, Placebo-Controlled, Parallel Group Study to Evaluate the Efficacy and Safety of Dupilumab in Patients With Persistent Asthma.

Q1: Lower quartile

Q3: Upper quartile

Abstract

Background:

With the introduction of different targeted therapies for T2 high asthma, there is an urgent need for markers to guide the choice of treatment. T2 high asthma includes different clinical phenotypes of asthma, but the prevalence and impact of activation of different T2 inflammatory pathways is unknown.

Objective:

We aimed to describe the level of co-expression of clinically available T2 inflammatory markers in patients with severe asthma, and the relationship with clinical characteristics and co-morbidities.

Methods:

Patients with severe asthma according to ERS/ATS guidelines were examined prospectively including sputum induction and grouped according to T2 biomarkers: blood eosinophilia ($\geq 0.3 \cdot 10^9/L$), total IgE (≥ 150 U/mL) and FeNO (≥ 25 ppb).

Results:

We found 116 (70%) of the 166 patients to have at least one T2 biomarker elevated: 39% had two or more elevated biomarkers while 31% had only one biomarker elevated. Concomitant airway- and systemic eosinophilia was present in 28% of all patients corresponding to half (53%) of the patients with either . Expression patterns of the T2 biomarkers were associated with differences in allergic sensitisation and the co-existence of nasal polyposis.

Conclusion:

The majority of patients with severe asthma showed at least one T2 inflammatory trait. Co-expression of T2 biomarkers was highly heterogeneous, and different expression patterns were associated with distinct clinical characteristics.

Introduction

Type 2 (T2) inflammation is considered the key inflammatory mechanism in asthma and is estimated to be present in roughly 50% of patients with severe asthma. Severe T2 high asthma is recognised as a heterogeneous entity and is considered to include several different clinical phenotypes of asthma(1). IL-5 and IL-4/13 are considered the key signalling pathways (2,3); IL-5 is the key cytokine for the recruitment and activation of eosinophils whereas IL-4 and IL-13 are the key cytokines driving the adaptive T2 immune response including the B cell immunoglobulin class switching observed in atopy (2).

The introduction of the biologic treatments targeting these pathways has elucidated their respective role and relation to the clinically available T2 biomarkers. The peripheral blood eosinophil count (B-EOS) reflects airway and systemic IL-5 production and is reduced markedly by anti-IL-5- but not anti-IL-4R α - therapy(4,5). In contrast, fractional exhaled nitric oxide (FeNO) is induced by IL-13(6), reflects airway IL-13 activity(7–9); and is markedly reduced by anti-IgE, anti-IL-13- and anti- IL-4R α -, but not anti-IL-5, therapy (4,5,10,11). Anti-IgE therapy reduced sputum eosinophil count (S-EOS) in early-phase clinical trials, but this was not verified in the phase 3 registration trials nor in a recent study of patients with uncontrolled severe atopic asthma (12–14).

With these targeted therapies has come an urgent need for markers that can guide the choice of treatment as well as predict and monitor treatment response. An important first step is uncovering the prevalence and implications of activity of more than one pathway as evidence suggest a possible additive effect. Concomitant activation of these pathways (as measured by B-EOS and FeNO) have been associated with increased exacerbation risk in moderate and severe asthma and a higher prevalence of impaired lung function, increased morbidity and increased exacerbation risk in mild-moderate asthma(15–18). Similarly, concomitant elevated B-EOS and IgE has been associated with increased exacerbation risk in moderate-to-severe asthma(19).

Currently, the relative importance of each T2 inflammatory pathways is unclear. For instance, suppressing eosinophils with anti-IL5 - without concomitantly suppressing FeNO - will generally reduce the number of exacerbations, in patients with severe eosinophilic asthma. Yet, the question of whether a high FeNO may signify a need for suppression of the IL-13 pathway as well remains unanswered..

With this study we aimed to describe the level of co-expression of the biomarkers used for subtyping of severe T2 high asthma. We hypothesised that we by describing the distribution of T2 biomarkers, and the relationship with the clinical patient profiles, would further our understanding of the heterogeneity of T2 inflammation and ultimately provide a stepping stone for future studies exploring treatment predicting biomarkers.

Methods

Study design

Data from two cross-sectional studies were pooled. Study 1: the SATS study (20), n=117 and Study 2: the SIGNATURE study (n=49).

In accordance with the ERS/ATS guidelines criteria on severe asthma, patients had to fulfil criteria of a physician's diagnosis of asthma and high-dose inhaled corticosteroid (ICS) treatment ($\geq 1600 \mu\text{g}$ budesonide) with a second controller (long-acting beta-agonist (LABA), theophylline or leukotriene-antagonist) for the previous year or oral corticosteroids (OCS) for $\geq 50\%$ of the previous year(1).

Exclusion criteria were: age below 18 years, pregnancy, sarcoidosis, malignant lung disease, known bronchiectasis without asthma or COPD, lung fibrosis, significant cardiac disease, lower airway infection requiring antibiotics or exacerbation within the previous 6 weeks requiring OCS, change in maintenance ICS-dose within the preceding 4 weeks.

Study population

All clinical examinations were conducted at the Respiratory Research Unit, Bispebjerg Hospital, Denmark. The SATS study included patients over a 12-month period from four Danish Respiratory outpatient clinics beginning October 2015. The SIGNATURE study included patients from Bispebjerg Hospital over an 18-month period beginning July 2017.

Clinical assessments:

Spirometry was conducted in accordance with ERS guidelines (21). A MasterScreenPneumo spirometer and MasterScreenBodyBox (BD Jaeger, Würzburg, Germany) were used for spirometry and body-box, respectively. Predicted values were calculated using the NHANES reference data (22).

In the SATS study, FeNO was measured using the Ecomedics CLD88sp (Ecomedics AG, Duernten, Switzerland) while the Niox Vero (Circassia Limited, Oxford, UK) was used in the SIGNATURE study (23). An elevated FeNO was defined as $\geq 25 \text{ ppb}$ (5,9,24–26).

B-EOS was assessed by the Bispebjerg Hospital pathology laboratory using automated flow cytometry with a Sysmex XN 9000 (Sysmex Europe GmbH, Norderstedt, Germany). Elevated B-EOS was defined as $\geq 0.3 \times 10^9/\text{L}$.

A total IgE above 150 U/mL was considered elevated. Specific serum IgEs (RAST test) was performed using a standard panel including pollen from birch (*Betula verrucosa*), grass (*Phleum pratense*) and mugwort (*Artemisia vulgaris*); dander from horse (*Equus caballus*), cat (*Felis domesticus*), and dog (*Canis familiaris*); house dust-mites: *Dermatophagoides pteronyssinus* and *Dermatophagoides farina*; and the fungi: *Alternaria alternata/tenuis* and *Cladosporium herbarum*. Allergic sensitisation was defined as elevated ($> 0.35 \text{ kU/L}$) specific IgE for a minimum one out of the 10 tested aeroallergens (27).

Airway hyperreactivity was assessed using the Mannitol test (Pharmaxis, Sydney, Australia) if $\text{FEV}_{1\%} \geq 70$.

177 Patients in the SIGNATURE study were examined using a nasal speculum and a history of prior or current
178 chronic rhinosinusitis with nasal polyposis was registered.

179 Self-reported co-morbidities, including chronic rhinosinusitis, atopic dermatitis gastroesophageal reflux was
180 addressed during the patient history. Self-reported asthma control was assessed using the five-item
181 Asthma Control Questionnaire (ACQ-5) (28).

182 An asthma exacerbation was defined as a course of OCS or an increase in maintenance OCS due to
183 worsening of symptoms.

184 **Sputum sampling:**

185 Sputum induction was done using hypertonic saline (3%, 4% or 5%). If FEV₁ was < 70% of predicted isotonic
186 saline was used (29).

187 **Sputum processing:**

188 Sputum was processed as described by Bafadhel et al. (30) and the cell differential count (CDC) was
189 conducted at the Respiratory Research Unit, Bispebjerg Hospital, Denmark.

190 Interpretation of sputum CDC

191 Cut-off values for eosinophilia and neutrophilia were $\geq 3\%$ and $\geq 61\%$, respectively (30,31). The following
192 inflammatory phenotype definition was used; Eosinophil asthma: eosinophils $\geq 3\%$ and neutrophils $< 61\%$;
193 neutrophil asthma: eosinophils $< 3\%$ and neutrophils $\geq 61\%$; mixed asthma: eosinophils $\geq 3\%$ and
194 neutrophils $\geq 61\%$ and paucigranulocytic asthma: eosinophils $< 3\%$ and neutrophils $< 61\%$ (32).

195 **Statistical analyses:**

196 SAS Enterprise guide version 7.1 was used for data analysis. Means (SDs) are presented for normally
197 distributed data and medians (quartile-1; quartile-3) are presented for non-parametric data. To correct for
198 multiple testing, the Bonferroni correction was employed and a p-value of 0.0025 was considered
199 significant in the between-group analyses.

200 Biomarkers with a right-tailed distribution were log-transformed to apply parametric analyses.

201 Patients were grouped based on elevation of IgE (≥ 150 U/mL), FeNO (≥ 25 ppb) and B-EOS ($\geq 0.3 \cdot 10^9/L$). We
202 grouped based on B-EOS rather than S-EOS to best reflect a routine clinical setting.

203 **Ethics:**

204 Both the SATS- (H-12014047) and the SIGNATURE study (H-17004938) was approved by the local Scientific
205 Ethics Committee and a written informed consent was signed by all patients.

206 **Results**

207 **Patient characteristics**

208 A total of 100 patients with severe asthma were included (table 1). Half (54%) of the patients were female,
 209 the mean age at diagnosis was 30 (20) years, and 26% of patients were diagnosed with asthma at or before
 210 the age of 12. Allergic sensitisation was present in roughly half (54%) of the patients.

211 The majority (72%) of patients had at least one elevated T2 biomarker, while 15% had all three T2
 212 biomarkers elevated (figure 1). Concomitant (39%) biomarker elevation was more prevalent than isolated
 213 (31%) biomarker increase and was equally prevalent for all pairwise combinations.

214 FeNO was the biomarker most prevalently elevated (44%), while IgE was elevated in 42% and 38% had
 215 blood eosinophilia. The median (Q1, Q3) of FeNO was 21 (12, 35), median IgE was 112 (29, 276) and the
 216 median blood eosinophil count was 0.21 (0.11, 0.41). Overall, 43% patients had airway eosinophilia
 217 (eosinophilic subtype: 40, mixed subtype: 32).

218 Two thirds (n=110, 66%) of patients were uncontrolled (ACQ5-score >1.5), and these patients were
 219 significantly older (p=0.05) and had smoked significantly less (p=0.04) compared to those with controlled
 220 disease.

221 Table 2 presents patient characteristics based on the presence of elevated B-EOS, IgE and FeNO, while table
 222 3 presents the expression of biomarkers across these groups. Adjusted for multiple comparisons, we only
 223 found significant differences across all groups in allergic sensitisation, S-EOS and the prevalence of the
 224 eosinophil sputum phenotype.

225 Allergic sensitisation was most prevalent in the groups with elevated IgE and was significantly less prevalent
 226 in the group with concomitantly elevated IgE and B-EOS compared with the group with concomitant
 227 elevated IgE and elevated FeNO (p=0.01) and the group with isolated elevated IgE (p=0.02).

228

229 Concomitant elevation of two or more biomarkers was associated with a higher prevalence of nasal
 230 polyposis. Three groups; the group with all biomarkers elevated, the group with concomitant elevated
 231 FeNO and blood eosinophilia and the group with concomitant elevated IgE and elevated FeNO; had a
 232 significantly higher prevalence of nasal polyposis compared with the group with isolated elevation of IgE
 233 (p=0.02, p=0.02 and p=0.05 respectively) and the group with no elevated biomarkers (p=0.002, p=0.001,
 234 p=0.02 respectively). Using logistic regression, we found FeNO (area under the curve (AUC) 0.86, p< 0.0001)
 235 and B-EOS (AUC 0.81, p< 0.003) to significantly predict nasal polyposis, whereas IgE (AUC 0.57, p< 0.45) did
 236 not. We found no significant difference between the receiver operating curves (ROC) for FeNO and B-EOS.

237 **Airway vs. systemic T2 inflammation**

238 A total of 134 (81%) patients had a sputum CDC and were included in the analyses of airway vs. systemic T2
 239 inflammation.

half (55%, n=36) of patients with eosinophilia (n=72) had concomitant airway- and systemic eosinophilia, whereas 21% (n=15) had isolated blood eosinophilia and 26% (n=19) had isolated sputum eosinophilia. Table 4 lists the clinical characteristics of these groups, while Table 5 lists biomarker expression.

Across the eosinophilic groups we found a significant difference in proportion of women (p=0.009), sputum neutrophil count (p=0.008) and in the proportion of patients with uncontrolled asthma (ACQ $\geq$ 1.5) (p=0.02).

S-EOS was significantly correlated to B-EOS (r=0.62, p<0.0001), FeNO (r=0.33, p<0.0001) and total IgE (r=0.28, p=0.001) and we found a good-to-excellent agreement between S-EOS and B-EOS (0.95, p=0.0001), FeNO (0.74, p=0.005) and IgE (72, p=0.001) using intra class correlation (ICC) (33).

We found a modest-to-fair agreement in the presence of T2 inflammation (using sputum eosinophilia ($\geq$ 0.3) as reference) using blood eosinophilia ($\geq$ 0.3) (kappa coefficient of 0.49, p<0.001), elevated FeNO ($\geq$ 25ppb) (kappa coefficient of 0.14, p=0.001) and elevated IgE ($\geq$ 75IE) (kappa coefficient of 0.34, p<0.001 (34,35).

Through multiple logistic regression sputum eosinophilia (S-EOS $\geq$ 3%) was significantly predicted by B-EOS (AUC 0.81, p<0.0001), FeNO (AUC 0.67, p<0.0001) and IgE (AUC 0.77, p<0.24) and B-EOS was significantly better than FeNO (p=0.01) but not IgE (p=0.42) at predicting sputum eosinophilia.

Discussion

In this unselected population of patients with stable severe asthma, we found 70% of patients to present a T2 inflammatory trait with a larger proportion having concomitant (39%) compared to isolated biomarker elevation (31%).

We found an overall discrepancy in the co-expression of IL-5- and IL-13 related biomarkers as only 44% of the patients with eosinophilia had concomitant sputum- and blood eosinophilia while the IL-13 induced biomarkers IgE and FeNO(36) only was co-elevated in 38% - a proportion equal to the prevalence of either with blood eosinophilia. B-EOS was the biomarker best reflecting airway eosinophilia, although only significantly better than IgE.

In summary, these findings underline the heterogeneity of T2 inflammation and support the existence of distinct subtypes of T2 high severe asthma.

At present, no studies have assessed the overlap in expression of FeNO, B-EOS and total IgE in patients with severe asthma albeit their pairwise overlap have been reported.. In a post hoc analysis of the DREAM-study; a double-blind, placebo-controlled trial assessing mepolizumab (anti-IL5) in patients with severe eosinophilic asthma (4); concomitantly elevated FeNO ($\geq$ 25ppb) and B-EOS ($\geq$ 0.15 $\cdot$ 10⁹/L) was found in 49% of patients with severe eosinophilic asthma while 42% of patients with moderate-to-severe asthma in the QUEST-study; a double-blind, placebo-controlled, trial assessing the efficacy of dupilumab (anti-IL4R α) in patients with uncontrolled moderate-to-severe asthma; had concomitant elevated FeNO ($\geq$ 25ppb) and B-EOS ($\geq$ 0.15 $\cdot$ 10⁹/L) (5,24). The prevalence found in our study was markedly lower (23%), most likely

explained by the lower cut-off for blood eosinophilia ($20.5 \cdot 10^9/L$ vs. $20.15 \cdot 10^9/L$) used in DREAM- and QUEST-study (4,24). Further, these studies were subject to a marked selection bias by excluding patients without exacerbations in the previous year (4,24). In a retrospective study of 884 patients with moderate-to-severe asthma (British Thoracic Society step 4 (70%) and step 5 (30%)) concomitant elevation of IgE (>75 kU/L) and B-EOS ($>0.4 \cdot 10^9$) was present in 33% of patients which is markedly higher than what we found (23%) (19). We attribute this difference to the inclusion of patients with moderate disease and the markedly lower cut-off for elevated IgE.

Contrary to the inverse relationship between T2- and non-T2 inflammation proposed by Choy et al. (37), we did not identify a solely neutrophilic group. Rather, our group without elevated T2 biomarkers was predominantly paucigranulocytic and were characterised by a well-preserved lung function, few exacerbations and a relatively low ACQ-5 score (table 3). We believe this points to a subpopulation of patients with a more benign and more steroid-responsive type of T2 inflammation.

Nasal polyposis was present in 40% of the assessed patients, and the presence of nasal polyposis was not exclusive for a single group(38). Expectedly, given the efficacy of anti-IL-5 and anti-IL-13 therapy on nasal polyposis, we found the prevalence of nasal polyposis to be significantly higher in patients with elevated FeNO (63% vs. 10%, $p<0.001$) and elevated B-EOS (63% vs. 17%, $p=0.001$) compared to those without any of these markers (39,40). Unlike Maniscalco et al. (grouping by B-EOS and FeNO), we found no significant differences between the groups with elevated T2 markers (38).

For FeNO, used 25 ppb as the cut-off as suggested by recent data and as used in the trials targeting IL-4R α (5,9,24–26). For B-EOS, used the cut-off identified in the MENSA trial ($0.3 \cdot 10^9/L$) and as employed by e.g. Bleecker et al. to evaluate treatment targeting the IL-5-receptor (41,42).

For IgE, we used a conservative cut-off of 150 U/mL, knowing that we would likely underestimate the proportion of patients with allergic driven elevation of IgE, as anti-IgE treatment is effective at much lower IgE levels(43,44). As in the U-BIOPRED- (126 U/mL) and SARP cohort (109 U/mL), the median level of IgE (112 U/mL) was, markedly above the normal range, and we believe that a lower cut-off would have resulted in less well-defined groups (45,46). Further, Garcia et al. found efficacy of anti-IgE therapy in patients with severe non-atopic asthma with a mean IgE of 153 U/mL supporting a cut-off 150 U/mL in order to reflect not only allergic driven disease(47).

Different methods of FeNO measurement were used in the two cohorts constituting the study population (SATS: Ecomedics CLD88sp, SIGNATURE: Niox Vero), but we do not believe this will introduce any particular bias given our cut-off of 25ppb, as the two methods have been found to be statistically equivalent for FeNO values < 70 ppb(48).

We found B-EOS to be significantly better than FeNO, but not IgE, in predicting sputum eosinophilia. This findings are in line with those of Wagener et al. in both a mild-moderate- and moderate-severe cohort (49), but are in contrast to those of Westerhof et al. as they reported both B-EOS and FeNO to be superior to IgE in identifying S-EOS in an unselected asthma population (50).

in 2014 Schleich et al. reported concordant (elevated) S-EOS and B-EOS in 19% of patients in a retrospective study of an unselected asthma population(51). We found similar proportions of patients without eosinophilia and with concomitant eosinophilia, but in contrast found the majority of patients with discordant B-EOS and S-EOS to have sputum eosinophilia rather than blood eosinophilia (51). We believe this difference is to be attributed to the difference in disease severity and thereby maintenance treatment.

While Schleich et al. found the group with concomitant eosinophilia to be male predominant and to have the lowest FEV₁ and the highest ACQ, FeNO and exacerbation rate, we found no significant differences across the groups, albeit the group with concomitant eosinophilia had a significantly higher FeNO than the group without eosinophilia ($p < 0.0002$).

A similar discrepancy was found in the IL-13 induced biomarkers IgE and FeNO (36) as co-elevation of these biomarkers was equally prevalent as either biomarker with B-EOS (eosinophilia $\cap$ IgE = 38%; eosinophilia $\cap$ FeNO = 38%). We consider this finding surprising, as IL-13 in conjunction with IL-4 induces IgE production in B-cells, as well as epithelial release of FeNO, and we would consequently expect a large degree of co-expression(36).

To date, the ability of T2 biomarkers to predict sputum eosinophilia has been interpreted as diagnostic accuracy (52) and has subsequently been used to rank the T2 biomarkers by performance as exemplified in the ERS consensus paper on severe eosinophilic asthma(53). Emerging evidence points to discordant and distinct roles of blood and airway eosinophils, as exemplified by the effect of anti-IL5 on the level of B-EOS and S-EOS (54,55), as well as on airway eosinophil activity by anti-IL-5(56). In fact, increasing evidence points to the importance of the site of the (eosinophil) inflammation(57) and in particular the work of Nair and Mukherjee have presented alternative inflammatory mechanisms in the patients with uncontrolled OCS dependent airway eosinophilic asthma (58,59).

Coverstone et al. recently summarized the FDA-approved biologic treatments for T2 high asthma and their biomarkers (44). In this context, 66% ($n=110$) of our patients were candidates (B-EOS $\geq 0.15 \cdot 10^9/L$) for Mepolizumab (anti-IL-5), Benralizumab (anti-IL-5R α) and Dupilumab (anti-IL-4R α); 26% ($n=43$) for (B-EOS $\geq 0.4 \cdot 10^9/L$) Reslizumab (anti-IL-5 α); and 58% ($n=97$) for (IgE 30-700 U/mL) Omalizumab (anti-IgE). Importantly, 49% ($n=68$) of patients with either B-EOS $\geq 0.15 \cdot 10^9/L$ or IgE 30-700 U/L had both elevated which further underlines necessity for a composite approach to biomarker analyses.

So far, unbiased clustering analyses have led to the identification of the clinical phenotypes of asthma currently recognised by the ERS/ATS(1,46,60,61) although the mechanisms (i.e. endotypes) underpinning the specific constellations of biomarkers identified in these studies remain largely uncovered.

Prediction models (e.g. multiple logistic regression) exploring these biomarker constellations will likely further our understanding of the interplay and relative importance of these biomarkers; however, in light of the heterogeneity of expression identified in this and other studies(62), we believe that inflammometry

at the molecular levels is a necessity in order to fully accommodate the complexity of the biology involved in T2 immunity.

In line with previous findings in more mild asthma, we found evidence suggesting a potential synergistic effect of pathway activation as the fifteen percent of patients who had all three T2 biomarkers concomitantly elevated had a significantly higher B-EOS ($p=0.03$) and FeNO ($p=0.006$), and a strong trend towards a higher IgE (0.06), compared with the respective groups with isolated increases. Further, the group had a significantly higher number of exacerbations than the group without an elevated T2 biomarker ($p=0.02$) and a strong trend towards a higher number of exacerbations compared with all the other groups combined ($p=0.06$), which is in line with the findings in patients with severe eosinophilic asthma from the DREAM-cohort (24).

Remarkably, 59% of these stable patients had blood eosinophilia and/or elevated FeNO despite high dose ICS treatment and/or OCS maintenance treatment. In a disease entity characterised by a large prevalence of steroid insensitivity this large prevalence of elevated steroid-predicting biomarkers is noteworthy, and questions whether these biomarkers still reflect steroid sensitivity. While this could be attributed to poor adherence, which has been reported in more than 50% of patients with suspected severe asthma (20), it could also reflect progression towards a more advanced state of disease and as such reflect a different mechanism than in steroid-responsive mild-moderate asthma.

The origin of this mechanism is unclear but could speculatively be attributed to either local auto-reactivity as a result of marked and continuous inflammation, or an innate immune response where group 2 innate lymphoid cells (ILC2) in particular have been highlighted as key mediators of largely steroid-resistant concomitant IL-4/13 and IL-5 driven inflammation (1,2,57,63–65). Future studies assessing inflammation at the molecular level are warranted to uncover these possible mechanisms.

In conclusion, we found three-fourths of patients to have an elevated T2 biomarker and interestingly only found a moderate level of co-expression of IL-5- and IL-13 related biomarkers respectively. Our findings illustrate the heterogeneity of T2 inflammation and support the existence of distinct subtypes of T2 high severe asthma. Our study reflects an unselected population of patients with stable severe asthma, and we believe the results to be generalisable and therefore relevant for both planning of future studies in severe asthma as well as in the development of treatment strategies at an organisational level.

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Figure legends

546 Figure 1.

547 **Title:** Overlapping expression of T2 biomarkers

548 **Legend:** Eosinophilia: blood eosinophil count $\geq 0.3 \cdot 10^9/\text{L}$; elevated FeNO: $\geq 25\text{ppb}$. Elevated IgE: $\geq 150 \cdot$
549 10^3 .

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Table 1:**Title:** Patient characteristics

Legend: ¥: only assessed in the SIGNATURE-study; SR= self reported; ♂: Allergic sensitization defined as positive specific IgE (RAST) for ≥1 of 10 standard allergens.

Study population	
N	166
Sex; n (%) female	90 (54)
Age; mean (SD)	49 (14)
Early onset (< 12 years); n (%)	34 (26)
BMI > 25; n (%)	48 (29)
Packyears; Median (Q1, Q3)	8 (1, 20)
ACQ5 score	1.9 (1.2, 3.0)
Uncontrolled asthma (ACQ5 >1.5); n (%)	110 (66)
ICS dose [µg of budesonide equivalent]; Median (Q1, Q3)	1600 (1600, 2400)
Use of prednisone for asthma; n (%)	15 (9)

Exacerbations previous 12 months	1 (0, 2)
Rhinosinusitis (SR); n (%)	143 (86)
Nasal polyps (¥); % of assessed (n assessed)	40 (48)
Allergic sensitization ¤; n (%)	90 (54)
Gastroesophageal reflux (SR); n (%)	58 (35)
Pre FEV ₁ %; mean (SD)	76 (21)
Pre FEV ₁ /FVC; mean (SD)	70 (16)
Mannitol †; % of tests positive (n tests)	47 (90)
Mannitol PD15; geometric mean (SD)	172 (2.7)
FeNO [ppb]; median (Q1, Q3)	21 (12, 35)
Total IgE [10 ³]; median (Q1, Q3)	112 (29, 276)
Blood eosinophils [10 ⁹] median (Q1, Q3)	0.21 (0.11, 0.41)
Sputum eosinophils [%] median (Q1, Q3)	2.0 (0.4, 9.1)
Sputum neutrophils [%] median (Q1, Q3)	50 (19, 73)
Sputum phenotype (n=164)	
Sputum; Eosinophil; n (%)	40 (24)

Sputum; Neutrophil; n (%)	17 (10)
Sputum; Mixed; n (%)	32 (20)
Sputum; Paucigranulocytic (%)	75 (46)

Table 2:

Title: Patient characteristics in subgroups based on the presence of elevated blood eosinophil count (Eos), IgE (IgE) and FeNO (FeNO)

Legend: A p-value of ≤ 0.0025 was considered significant to correct (Bonferroni) for multiple comparisons. †Mannitol challenge test performed if FEV1 > 70% of predicted. #:Kruskal wallis, §: Chi-square, ‡:Fishers exact test, †Welch's ANOVA, µg of budesonide equivalent;

	Group 1 None	Group 2 Eos + IgE + FeNO	Group 3 Eos + FeNO	Group 4 FeNO + IgE	Group 5 Eos + IgE	Group 6 FeNO	Group 7 IgE	Group 8 Eos	P
N	50	25	13	14	13	21	18	12	
Sex; %-female	64	32	62	21	54	76	56	50	0.01 §
Age; mean (SD)	44 (13)	51 (14)	54 (10)	53 (13)	47 (14)	54 (14)	48 (14)	50 (17)	0.05 †
Early onset; n (%)	26	24	8	14	31	19	44	41	0.30 †
Overweight; %	26	24	15	36	23	38	50	17	0.40 †
Packyears; Median (Q1, Q3)	5 (1, 15)	10 (0, 19)	13 (3, 20)	3 (1, 6)	13 (0, 20)	13 (2, 28)	15 (4, 25)	11 (0, 38)	0.63 #
ACQ5 score	1.8 (1.2, 2.6)	2.2 (1.4, 3.0)	3.0 (1.8, 3.4)	2.6 (2.2, 3.4)	1.6 (1.0, 2.0)	1.8 (1.0, 2.6)	2.3 (1.0, 3.2)	1.8 (0.9, 2.3)	0.02 #
Uncontrolled asthma (ACQ5 >1.5); %	58	64	92	86	54	71	67	58	0.21 †
ICS dose; Median (Q1, Q3)	1800 (1600, 2400)	1600 (1600, 2400)	1600 (1600, 2000)	1600 (1600, 1600)	1600 (1600, 1600)	1600 (1600, 2400)	1600 (1600, 2000)	3200 (2400, 3200)	0.08 #
Use of prednisone for asthma; %	4	4	15	7	8	14	22	8	0.13 †
Exacerbations previous 12 months; median [Q1, Q3]	0 (0, 1)	2 (0, 3)	2 (0, 2)	0 (0, 1)	1 (0, 2)	1 (0, 3)	1 (0, 3)	1 (0, 2)	0.11 #
Pre FEV₁ %; mean (SD)	85 (21)	72 (21)	69 (14)	80 (21)	62 (208)	76 (19)	73 (19)	72 (29)	0.01 †
Pre FEV₁/FVC; mean (SD)	74 (12)	67 (11)	64 (90)	71 (14)	60 (15)	72 (11)	75 (31)	66 (16)	0.02 †
Mannitol †; % of tests positive (n with a test))	38 (34)	58 (12)	88 (8)	40 (10)	25 (4)	56 (9)	43 (7)	33 (6)	0.28 †
Mannitol PD15; geo mean (95CI)	165 (23, 613)	190 (49, 630)	171 (14, 491)	144 (41, 428)	355 (na.)	162 (67, 530)	302 (205, 462)	83 (33, 211)	0.73 †

Table 3:

Title: Biomarkers among subgroups based on the presence of elevated blood eosinophil count (B-EOS), IgE (IgE) and FeNO (FeNO)

Legend: A p-value of ≤ 0.0025 was considered significant to correct (Bonferroni) for multiple comparisons. ‡: Fishers exact test, †Welch's ANOVA.

	Group 1 None	Group 2 EOS + IgE + FeNO	Group 3 EOS + FeNO	Group 4 FeNO + IgE	Group 5 EOS + IgE	Group 6 FeNO	Group 7 IgE	Group 8 EOS	P
N	50	25	13	14	13	21	18	12	
FeNO [ppb]; median (Q1, Q3)	12 (9, 19)	52 (33, 66)	47 (42, 60)	31 (27, 42)	13 (12, 19)	33 (28, 44)	14 (9, 19)	12 (8, 17)	
Total IgE [10³]; median (Q1,Q3)	26 (8, 88)	721 (260, 1420)	75 (54, 80)	337 (219, 710)	384 (206, 921)	48 (13, 85)	323 (184, 786)	52 (19, 116)	
Blood eosinophils [10⁹], median (Q1,Q3)	0.11 (0.09, 0.16)	0.52 (0.43, 0.70)	0.52 (0.38, 0.75)	0.22 (0.19, 0.26)	0.45 (0.38, 0.72)	0.12 (0.09, 0.21)	0.14 (0.09, 0.20)	0.42 (0.35, 0.52)	
Allergic sensitization ⌘; %	42	76	31	93	46	33	89	33	<0.001 †
Sputum eosinophils [%], median (Q1, Q3)	0.5 (0.0, 1.5)	17.5 (5.0, 42.4)	14.4 (0.8, 76.0)	2.3 (0.8, 7.0)	6.9 (2.6, 48.6)	1.6 (0.3, 2.5)	2.3 (0.3, 7.0)	6.9 (1.0, 12.1)	<0.001 †
Sputum neutrophils [%], median (Q1, Q3)	48 (37, 71)	40 (9, 55)	29 (8, 53)	53 (26, 60)	39 (17, 82)	70 (20, 86)	59 (35, 79)	45 (19, 81)	0.26 †
Sputum phenotype;									
Eosinophil (%)	2	63	38	36	38	5	29	25	<0.001 ‡
Neutrophil (%)	24	4	8	14	8	43	29	8	0.02 ‡
Mixed (%)	6	8	8	0	31	14	6	25	0.05 ‡
Paucigranulocytic (%)	68	25	46	50	23	38	35	42	0.02 ‡

Table 4:

Title: Patient characteristics in subgroups based on the presence of elevated blood- and sputum eosinophil count

Legend: A p-value of ≤ 0.0025 was considered significant to correct (Bonferroni) for multiple comparisons. †Mannitol challenge test performed if FEV1 > 70% of predicted. #:Kruskal wallis, §: Chi-square, ‡:Fishers exact test, †Welch's ANOVA. NA: not applicable.

	Group 1 None	Group 2 B-EOS and S-EOS	Group 3 S-EOS	Group 4 B-EOS	P
N	62	38	19	15	
Sex; %-female	68	37	37	60	0.009 §
Age; mean (SD)	48 (14)	53 (14)	56 (11)	43 (12)	0.01 †
Early onset; n (%)	18	21	32	40	0.001 ‡
Overweight; %	39	13	32	33	0.0002 ‡
Packyears; Median (Q1,Q3)	8 (1, 20)	13 (2, 20)	7 (3, 18)	6 (0, 13)	0.43 #
ACQ5 score	2.0 (1.2, 2.8)	1.8 (1.2, 3.0)	2.6 (1.8, 3.6)	1.7 (1.0, 3.0)	0.18 #
Uncontrolled asthma (ACQ5 >1.5); %	68	66	74	60	0.86 ‡
ICS dose; Median (Q1, Q3)	1700 (1600, 2400)	1600 (1600, 2400)	1600 (1600, 2400)	1600 (1600, 1600)	0.48 #
Use of prednisone for asthma; %	8	10	21	0	0.03 ‡
Exacerbations previous 12 months; median [Q1, Q3]	1 (0, 2)	1 (0, 3)	0 (0, 3)	1 (1, 2)	0.85 #
Pre FEV₁ %; mean (SD)	82 (20)	67 (22)	74 (20)	75 (20)	0.009 †
Pre FEV₁/FVC; mean (SD)	73 (12)	62 (13)	74 (31)	70 (14)	0.01 †
Mannitol †; % of tests positive (n with a test))	46 (37)	6 (15)	25 (8)	30 (10)	0.18 ‡
Mannitol PD15; geo mean (95CI)	160 (67, 308)	210 (140, 356)	166 (40, 293)	200 (150, 440)	0.09 †

Table 5:

Title: Biomarkers in subgroups based on the presence of elevated blood- and sputum eosinophil count

Legend: A p-value of ≤ 0.0025 was considered significant to correct (Bonferroni) for multiple comparisons. #:Kruskal wallis, §: Chi-square, †Welch's ANOVA. NA: not applicable.

	Group 1 None	Group 2 B-EOS and S-EOS	Group 3 S-EOS	Group 4 B-EOS	P
FeNO [ppb]; median (Q1, Q3)	20 (10, 28)	31 (13, 52)	21 (15, 44)	35 (15, 65)	0.003 †
Total IgE [10 ³]; median (Q1, Q3)	64 (16, 143)	290 (131, 1100)	263 (182, 637)	151 (67, 264)	0.001 †
Blood eosinophils [10 ⁹] median (Q1, Q3)	0.12 (0.10, 0.18)	0.52 (0.38, 0.88)	0.21 (0.11, 0.26)	0.46 (0.35, 0.60)	NA
Allergic sensitization ♂; %	47	53	63	53	0.66 §
Sputum eosinophils [%], median (Q1, Q3)	0.5 (0.0, 1.5)	18.0 (7.3, 61.8)	7.3 (4.3, 29.3)	0.75 (0.3, 1.3)	NA
Sputum neutrophils [%], median (Q1, Q3)	54 (27, 81)	38 (9, 67)	53 (19, 69)	42 (16, 63)	0.14 †
Sputum phenotype;					
Eosinophil (%)		74	63		
Neutrophil (%)	45			27	
Mixed (%)		26	37		
Paucigranulocytic (%)	55			73	

