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TITLE: EFFECTIVENESS OF BENRALIZUMAB IN SEVERE EOSINOPHILIC ASTHMA:
DISTINCT SUB-PHENOTYPES OF RESPONSE IDENTIFIED BY CLUSTER ANALYSIS.

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19 **KEY MESSAGES**

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21 Benralizumab is effective in severe eosinophilic asthma, but it is not successful in all patients, and
22 suboptimal responses are reported.

23

24 This study suggests the presence of different clusters within the apparently homogeneous severe
25 eosinophilic asthma Type2-phenotype.

26

27 These clusters, characterized by differential expression of type-2 biomarkers and distinct clinical
28 features, show differential benralizumab responsiveness.

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30

1 ABSTRACT

2 **Background:** Benralizumab is effective in severe eosinophilic asthma (SEA), but suboptimal
3 responses are observed in some patients. Although several factors have been associated with
4 benralizumab response, no cluster analysis has yet been undertaken to identify different
5 responsiveness sub-phenotypes.

6 **Objective:** To identify SEA sub-phenotypes with differential responsiveness to benralizumab.

7 **Methods:** One hundred-five patients diagnosed with SEA who had completed 6 months of
8 benralizumab treatment were included in a hierarchical cluster analysis based on a set of clinical
9 variables that can be easily collected in routine practice (age, age at disease onset, disease length,
10 allergen sensitization status, blood eosinophil count, IgE levels, FEV₁% predicted, nasal polyposis,
11 bronchiectasis).

12 **Results:** Four clusters were identified: Clusters 2 and 3 included patients with high levels of both
13 IgE and eosinophils (Type-2 biomarkers high), whereas Clusters 1 and 4 included patients with
14 only one Type-2 biomarker at a high level: IgE in Cluster 1 and eosinophils in Cluster 4. Clusters
15 2 and 3 (both Type-2 biomarkers high) showed the highest response rate to benralizumab in terms
16 of elimination of exacerbations (79% and 80% respectively) compared to Clusters 1 and 4 (52%
17 and 60%, respectively). When super-response (absence of exacerbation without oral corticosteroid
18 use) was assessed, Cluster 2, including patients with more preserved lung function than the other
19 clusters, but comparable exacerbation rate, oral corticosteroid use and symptom severity, was the
20 most responsive cluster (87.5% of patients).

21 **Conclusions:** Our cluster analysis identified benralizumab differential response sub-phenotypes in
22 SEA, with the potential of improving disease treatment and precision management.

23

1 INTRODUCTION

2 Benralizumab has been shown to be effective in patients with severe eosinophilic asthma (SEA).¹⁻⁴
3 Phase 3 randomized controlled trials (RCTs) have demonstrated a significant reduction in
4 exacerbation rate and oral corticosteroid (OCS) use, as well as improvement of lung function,
5 asthma control and quality of life measures.¹⁻⁴ However, as for other biologics, it is evident that
6 not all patients respond equally well.⁵ This difference in treatment response is likely multifactorial
7 and related to the heterogeneity of the severe asthma population.⁶
8 Prediction of treatment response is difficult and deals with various problems such as how to define
9 a response, what are clinically relevant outcome measures, and what should be the timing for the
10 evaluation of response.⁷ Data from RCTs consistently showed that higher levels of blood
11 eosinophils and a history of frequent exacerbations were the most important baseline
12 characteristics that predicted treatment response to benralizumab.⁸ The presence of late-onset
13 asthma (>18 years), OCS dependency, impaired lung function and nasal polyposis might further
14 increase the chance of a good response.⁹
15 It is recognized that subjects recruited in RCTs are not fully representative of the severe asthma
16 population, as seen in routine practice, since trial populations appear to have a moderate-to-severe
17 disease.^{1,10-13} Furthermore, patients with fixed-flow obstruction, smoking history and important
18 medical comorbidities are usually excluded.¹⁴ Consequently, real-life data on biologics are needed
19 to assess effectiveness and identify clinically relevant predictors, as data generated in clinical trial
20 settings may not be easily translated in a real-life setting.
21 In this multicenter, observational, retrospective study, by cluster analysis, we sought to
22 characterize different clinical sub-phenotypes within SEA, aiming at identifying subgroups of
23 patients with differential benralizumab responsiveness.

1 METHODS

2 *Study design*

3 We performed a retrospective analysis of all patients with SEA who commenced treatment with
4 benralizumab between May 2019 to January 2020 at 12 Italian tertiary referral asthma Centers.
5 Informed consent for the retrospective analysis was obtained from all subjects. The involved
6 asthma centers used a shared anonymized database to collect clinical, functional, and biological
7 data. Ethical approval was obtained from each local Ethic Committee (Coordinator center:
8 Catania, Italy; document number, 33/2020/PO – 14th/April/2020).

9 *Study population*

10 All patients were reviewed by expert physicians and fulfilled the ATS/ERS definition of severe
11 asthma.¹⁴ All patients met spirometric criteria for asthma. Patients started benralizumab if they had
12 an eosinophil count $\geq 300/\mu\text{L}$ in the preceding 12 months and a minimum of 2 exacerbations in the
13 previous year and/or were on maintenance oral corticosteroid (mOCS).¹⁵ Exacerbations were
14 defined as a worsening in asthma control requiring ≥ 3 days of oral prednisone treatment or
15 increase of prednisone dose if already on mOCS. Baseline exacerbation rate was determined by
16 reviewing patients' medical records. Patients' demographic data were collected from the patient's
17 clinical file.

18 *Outcomes and analysis*

19 Clinical assessment was performed at each scheduled visit. Complete data were collected at T6
20 and T12 (6 and 12 months after benralizumab, respectively). Data collected included: number of
21 exacerbations, oral corticosteroid dosage, asthma control test (ACT),¹⁶ mini-asthma quality of life
22 questionnaire (mini-AQLQ),¹⁷ Sino-nasal Outcome Test-22 (SNOT-22),¹⁸ spirometry, peripheral
23 blood eosinophil count, total IgE levels, fractional exhaled nitric oxide (FeNO).

24 Clinical response was defined for each outcome as follows: 1) $\geq 50\%$ reduction in the exacerbation
25 rate (ER); 2) $\geq 50\%$ reduction in mean daily OCS dose; 3) $\geq 0.23\text{L}$ increase of pre-bronchodilator
26 FEV1 or FEV1 $\geq 80\%$;^{19,20} 4) ≥ 3 point increase in ACT score or ACT score ≥ 20 ;²¹ 5) ≥ 0.5 point
27 increase in mini-AQLQ score.¹⁻⁴

28 A further analysis was performed in a subgroup of patients defined as "super-responders",
29 representing subjects who were completely exacerbation-free and no longer required OCS
30 (ER0/OCS0). Two additional sub-groups were also defined within the super-responders: 1) those
31 with ACT score ≥ 20 (ER0/OCS0/ACT ≥ 20); 2) those with ACT score ≥ 20 and FEV1 $\geq 80\%$
32 (ER0/OCS0/ACT ≥ 20 /FEV1 ≥ 80).

1 Patients who did not show a 50% reduction in exacerbation rate or mOCS use were defined as
2 “non-responders”. However, in all the analyses performed in this study, they were included in the
3 “non-superResponders” group, as opposite to “superResponders”.

4 5 *Statistics*

6 Data were analysed by means of SPSS software (IBM SPSS, Chicago, IL). Data were shown as
7 mean and standard deviation (SD) if normally distributed, or median and interquartile range (IQR)
8 if not. To test the normality of distributions, the Shapiro-Wilk test was used. Categorical variables
9 were analysed by Chi-Square test or Fisher’s exact test when appropriate.

10 To compare the 3 measurements at T0, T6 and T12, respectively, the Friedman test was used.

11 Alternatively, the Wilcoxon test for paired samples was used when investigating the possible
12 differences between paired medians.

13 The Ward's hierarchical clustering method (squared Euclidean distance) was used. Moreover, a k-
14 means clustering was carried out. The optimal number of clusters detected through the hierarchical
15 cluster approach was confirmed as such by means of the Silhouette coefficient and the scree plot.

16 Clusters’ comparisons were performed using one way Anova test for continuous variables and the
17 Marascuilo test for multiple proportions (independent samples).

18 The t Student test and the non-parametric Mann-Whitney U test were used to compare the two
19 unrelated samples corresponding to SuperResponders/Non-SuperResponders.

20 Predictive analyses on responders were conducted using logistic regression (Ward’s criterion).

21 Variable were selected through the backward elimination process and considered significant at
22 $p \leq 0.05$.

23 Differences were considered significant at $p < 0.05$ and all the values are two-sided.

1 RESULTS

2 A total of 160 patients who commenced benralizumab were included in this analysis. The baseline
3 patient characteristics (68.1% females; mean age of 53.8 ± 13.9 years; BMI, 26.6 ± 5.1) are shown in
4 Table 1. The median peripheral blood eosinophil count was 690 cells/ μ L (IQR, 460; 975), the
5 median FeNO concentration was 45 ppb (IQR, 24.7; 62.2), the median IgE level was 134 kU/L
6 (IQR, 57; 368). Furthermore, other eosinophilic comorbidities were reported, such as nasal
7 polyposis (49.4% of patients) and atopic dermatitis (10.6% of patients). Bronchiectasis was
8 present in 21.9% of cases. Sixty percent of patients were sensitized to inhaled allergens. Despite
9 all patients were prescribed high dosage ICS/LABA, with 59.4% of them on mOCS, the control
10 was poor (ACT, 13.6 ± 4.1 ; AQLQ, 3.4 ± 1.1 ; mean exacerbations in the 12 months prior to
11 benralizumab, 5 ± 3.4) and mean pre-bronchodilator FEV₁% predicted was 65.6 ± 21.1 .
12 After 6 months of treatment, overall disease control was significantly improved, with reduction in
13 exacerbations and OCS use with a parallel improvement in lung function, ACT and AQLQ scores
14 (Table 2), as well as a reduction in FeNO concentration and eosinophil count (Table 2). The
15 benefit observed after 6 months remained stable for the following months (T12) (Table 2).
16 At T6, 111 out of 126 patients (88.1%) were responders, defined as having $\geq 50\%$ reduction in ER
17 and $\geq 50\%$ reduction in mean daily OCS dose. As for super-responders, 84 (65.1%) patients
18 remained completely exacerbation free at T6, without requiring mOCS (ER0/OCS0).
19 Thus, despite a general good response to benralizumab, about 1/3 of patients had a suboptimal
20 response, as they did not eliminate either exacerbations or mOCS (non-SuperResponders).
21 We, thus, tried to identify sub-phenotypes of patients with differential responsiveness to
22 benralizumab.
23 In order to explore/investigate patients' characteristics in depth, a hierarchical cluster analysis was
24 carried out using a set of variables that can be easily collected in routine practice. They included:
25 age, age at disease onset, disease length, family history of atopy, allergen sensitization (Skin Prick
26 Test positive/negative), blood eosinophil count, IgE levels, FEV₁% predicted, nasal polyposis,
27 bronchiectasis, number of exacerbations in the previous year. Moreover, a k-means clustering was
28 carried out and the number of 4 clusters detected through the hierarchical cluster approach was
29 confirmed as optimal (Supplementary-Figure-1).
30 Clustering the 105 available patients at T6 with our hierarchical algorithm (21 out of 126 patients
31 were excluded because of $\geq 10\%$ of missing data in at least one of the variables used for

clustering) revealed four distinct asthma clusters (Table 3). The 12 month time point could not be used because the number of patients observed was too small (n=49).

Cluster 1 (less eosinophilic, allergic [eosinophils ↓, IgE ↑], older, low nasal polyps frequency, impaired lung function) included 23 patients characterized by the high percentage of allergic sensitization (SPT positive for both perennial and/or seasonal allergens, 100%) who underwent allergen immunotherapy (AIT) (30.4%) and the lowest peripheral median eosinophil count (470 cells/ μ L). This cluster included patients with the oldest disease onset (46.3 ± 8.4 years).

Cluster 2 (type-2 biomarkers high [eosinophils ↑, IgE ↑], young, preserved lung function), including 19 patients, was characterized by the youngest age of patients under treatment and the youngest patients' age at disease onset. Furthermore, patients in cluster 2 showed the highest level of Type 2 biomarkers, such as peripheral blood eosinophil count, FeNO and IgE levels. The lack of statistical significance in FeNO levels between the groups might be due to the high number of missing observation for this variable. Patients in this subgroups featured also less functional impairment (FEV₁% predicted, 79.3%) and were never treated with azithromycin.²²

Cluster 3 (type-2 biomarkers high [eosinophils ↑, IgE ↑], older, exacerbating, nasal polyps, bronchiectasis, impaired lung function), including 35 patients, was characterized by the longest mean disease duration, the highest mean exacerbation number in the year prior to benralizumab (8.4 ± 3.9) and the highest frequency of nasal polyps and bronchiectasis (these latter features were not statistically different between the clusters).

Cluster 4 (eosinophilic, non-allergic [eosinophils ↑, IgE ↓], older, nasal polyps, impaired lung function) included 28 patients, mainly non-allergic (SPT positive, 7.1%) and without a family history of atopy (25%) with the lowest total IgE levels (median=68 kU/L) and old age at disease onset (44.9 ± 11.9 years).

These 4 clusters showed differential benralizumab responsiveness after 6 months of treatment.

(Figure 1). Cluster 2 (n=19) and cluster 3 (n=35) included the greater percentage of patients who remained exacerbation free after benralizumab (ER0) (Figure 1, A). Furthermore, Cluster 2 showed overall the strongest effect of benralizumab with the highest rate of super-response according also to all the 3 remaining criteria (ER0/OCS0; ER0/OCS0/ACT $\geq$ 20; ER0/OCS0/ACT $\geq$ 20/FEV₁ $\geq$ 80%) (Figure 1, A, B, C, D).

Ten patients out of the 105 patients included in the cluster analysis were also affected by EGPA or eosinophilic pneumonia. In order to assess benralizumab responsiveness in a purer SEA population, a further analysis after the exclusion of these patients was performed. As shown in

1 Supplementary-Figure 2, the rate of super-responders ER0/OCS0 in Cluster 3 increased from 66%
2 to 74%.
3 Baseline variables associated to super-response (ER0, OCS0) at T6 were also explored comparing
4 super-responders and non-superresponders, regardless their cluster distribution. The chance of
5 gaining a super-response (ER0, OCS0) was significantly associated with lower rate of OCS-
6 dependency, lower leukotriene receptor antagonists (LTRA) use and the presence of nasal
7 polyposis (Table 4). However, multivariate logistic regression analysis confirmed only nasal
8 polyposis, as independent predictor of super-response. Older age, younger disease onset and
9 absence of azithromycin treatment were also identified as independently associated with the
10 chance of being super-responder (ER0/OCS0) (Table 5). Thus, 3 out of 4 independent predictors
11 identified by logistic regression analysis, namely age, age at disease onset and azithromycin use,
12 were also differently expressed in the clusters. This indirectly confirms the validity of the
13 clustering process. FeNO was not entered in the logistic regression model due to high number of
14 missing data.

1 DISCUSSION

2 To investigate differential benralizumab responses among patients with severe eosinophilic asthma
3 and the various factors that contribute to them, we performed a cluster analysis based on a set of
4 clinical variables and inflammatory biomarkers that can be easily collected in the routine practice.

5 This approach identified four distinct clusters within adult-onset SEA patients with variable
6 benralizumab responsiveness.

7 Patients with SEA experience frequent exacerbations. These patients often depend on the chronic
8 use of OCS or use OCS during exacerbations, with increased risk of OCS-related adverse events.

9 Therefore, especially in real-life settings, when assessing responsiveness to biologics, the
10 elimination of OCS should be considered as a critical outcome. Thus, using a response definition
11 as either 50% reduction in exacerbations (with consequent reduction of OCS) or 50% reduction in
12 mOCS dose for OCS-dependent patients might be not completely adequate. According to this
13 definition of response we observed 88.1% response rate in our cohort, showing that benralizumab
14 is highly effective. However, this percentage decreased to 65,1% when responders were
15 considered those patients who eliminated both exacerbations and OCS use (superresponders,
16 ER0/OCS0). Thus, about 1/3 of patients had a suboptimal response, despite what appeared to be
17 an appropriate eosinophilic phenotype, with a median peripheral eosinophil count of 690 cell/ μ L
18 (IQR, 460; 975) in the year prior to benralizumab.

19 Our findings showed that distinct characteristics related both to the level of inflammation (showed
20 by the combination of 2 inflammatory biomarkers, namely eosinophil count and IgE levels) and to
21 disease history and comorbidities may contribute to differential benralizumab responsiveness
22 observed in the 4 clusters.

23 Cluster 2 and 3 showed the highest response rate (compared to cluster 1 and 4) when response was
24 assessed as elimination of exacerbations (ER0) (Figure 1, A). These clusters included patients with
25 high levels of both peripheral blood eosinophils and IgE (Table 3). In contrast, Clusters 1 and 4,
26 characterized by only one T2-biomarker high (IgE only for Cluster 1, and eosinophils only for
27 Cluster 4) showed a lower ER0 response rate (Figure 1-A). Notably, ER0 response rate was
28 comparable between Clusters 2 and 3, (around 80%) despite Cluster 2 contained subjects with
29 fewer exacerbations (3.6 ± 1.5 exacerbations/patient), while Cluster 3 contained patients with the
30 most exacerbations in the previous year (8.4 ± 1.5 exacerbations/patient) (Table 1). Collectively
31 these data may suggest that elimination of exacerbations is correlated with a high level of Type-2
32 inflammation, which can be assessed as the presence of two biomarkers (namely eosinophils and

IgE) at high level (Clusters 2 and 3). The pooled analysis of the SIROCCO and CALIMA studies showed a correlation between enhanced benralizumab efficacy in exacerbation reduction and increased peripheral eosinophil count, as a biomarker of Type-2 inflammation.⁸ However, our data show that increased eosinophil levels alone are not sufficient to identify highly ER0 responsive sub-phenotypes. In fact, Cluster 4, characterized by high eosinophil count (but low IgE level), showed a lower ER0 response rate. Univariate analysis and logistic regression analysis seem to support this consideration, as eosinophil counts were not independently associated with a super-response (Table 4 and Table 5). Thus, a combination of Type-2 biomarkers (eosinophils and IgE) seems more sensitive in identifying subjects with higher chance of exacerbation reduction.

Cluster 2 showed overall the strongest effect of benralizumab. In fact, Cluster 2 was also the most responsive cluster when super-response was assessed, using the 3 pre-specified super-response criteria (ER0/OCS0; ER0/OCS0/ACT $\geq$ 20; ER0/OCS0/ACT $\geq$ 20/FEV₁ $\geq$ 80%) (Figure 1, B, C, D). However, when a pure asthmatic population was analyzed, after the exclusion of patients with EGPA and eosinophilic pneumonia, the ER0/OCS0 super-response rate in Cluster 3 was increased, (it did not reach Cluster 2 super-response rate, but it was higher than Cluster 1 and 4 super-response rate), confirming that patients with both Type-2 biomarkers high tend to have a better response, despite their different severity at baseline (Supplementary-Figure 2).

Notably, although lung function of Cluster 2 patients was more preserved (therefore, this might have been considered a less severe group at baseline), exacerbation number, OCS use, ACT, AQLQ, and SNOT-22 were comparable to that of Cluster 1 and 4. Thus, patients in Cluster 2 showed a clinical picture similar to that of clusters 1 and 4 (comparable severity).

The different super-response rate between Clusters 2 and 3 (Figure 1-B,C,D), despite their comparable ER0 response rate (Figure 1-A) deserves some considerations.

Although Clusters 2 and 3 are both characterized by high Type-2 inflammation (high levels of eosinophils and IgE) and comparable age at onset, the higher frequency of nasal polyps and bronchiectasis in Cluster 3 might identify a different sub-phenotype. Furthermore, patients in Cluster 2 never used azithomycin, whereas Cluster 3 featured the highest proportion of patients who had been treated with azithromycin, probably because of their highest exacerbation rate.²²

Finally, the lower lung function of subjects in Cluster 3 could be the result of a longer disease with repeated exacerbations, since patients in Cluster 3 have a longer disease duration than those in Cluster 2 (Table 3). Thus, besides high level of Type-2 biomarkers, also clinical features and disease duration or age at onset, may contribute to identify bernalizumab super-responsiveness

sub-phenotypes. In other words, it is possible that clusters 2 and 3 included similar patient populations (as suggested by their comparable levels of inflammatory markers) that received treatment at different phases of their disease, as Cluster 3 included patients with longer disease duration (who commenced treatment at older age) (Table 3). The importance of age at onset was confirmed by logistic regression analysis (Table 5). In conclusion, older age at onset and the presence of only one inflammatory biomarker at high level (either blood eosinophil or IgE) are characteristics shared by the two lower response Clusters 1 and 4, according to all 4 response criteria. In contrast, younger age at onset and high levels of the 2 inflammatory biomarkers characterize the highest ER0 response rate Clusters 2 and 3 (Table 3; Figure 1, A). Data from RCTs and meta-analyses on biologics in severe asthma showed that there is limited evidence for improvement in health-related quality-of-life scores (e.g. ACT, AQLQ) and lung function, which may not meet clinically detectable levels.²³ We confirm in this series that the proportion of patients achieving a clinically meaningful symptom reduction (ACT) and improvement of lung function (FEV₁%) is generally small (Figure 1, C-D). However, a high proportion of patients with a clinically meaningful improvement of ACT and FEV₁ was shown in Cluster 2. Notably, ACT at baseline of Cluster 2 patients was comparable to that of patients included in the other clusters.

Limitations and strengths

Despite the relatively small patient number for a cluster analysis, the hierarchical clustering method used appeared to be sensitive in identifying clusters with differential bernalizumab responsiveness. In particular Cluster 2 seems to be well differentiated from the other clusters, as response is always the highest according to all the 4 criteria used. This analysis has the inherent limitations of all the retrospective studies. In particular, the high number of missing data prevented the use of some variables, namely FeNO, ACT or AQLQ, in the generation of clusters.^{24,25} This might have influenced the composition of the cluster themselves. Validation/replication of our data in different patient populations will be useful to confirm and expand our findings, particularly for importance of FeNO as inflammatory biomarker.²⁶⁻²⁸ Our data confirmed that IgE alone are not predictive of response to bernalizumab. However, high levels of IgE seem to be predictive of response if associated to high levels of eosinophils. We speculated that the association of different T2 biomarkers (such as IgE, eosinophils, FeNO) might identify more responsive subgroups. In this light, an important limitation of this study is that FeNO was not available for the analysis owing to numerous missing data. Notably, Cluster 2, the

1 most responsive one, besides high levels of IgE and eosinophils, showed also the highest FeNO
2 levels, but unfortunately this biomarker could not be considered in our analysis.
3 Patients started treatment with benralizumab if they complained persistent respiratory symptoms
4 despite regular treatment with high dosages of ICS/LABA, with or without LRTA or LAMA or
5 OCS. However, since this is a real-life study, no measure of drug compliance before entering the
6 study was carried-out.

7 We are confident that patients' data collection was quite accurate, as all patients were examined
8 regularly in the outpatient clinic of the Centers involved in the study. However, as for all the real-
9 life studies, it is possible that a limited amount of information was missed since some patients
10 might have accessed different healthcare settings during their exacerbations.

11 The 6-month evaluation time point could be considered not fully adequate to assess exacerbation
12 rate and other outcomes. However, the effects of benralizumab remained stable in the following
13 months (T12) (Table 2), suggesting that results observed after six months of benralizumab are
14 representative of later time-points.

15 Considering the general prevalence of depression in asthmatic patients (11-13%), it is possible that
16 depression was underestimated in this series (4%).^{29,30} It could be related to some drawbacks of
17 the Beck Depression Inventory (BDI), used for diagnosis of depression in this real-life series (e.g.
18 the BDI has no safeguards against faking, lying, or variable response sets; the BDI is extremely
19 sensitive to differences in the instructions given to an examinee).³¹

20 Although we used univariate and logistic regression analyses to indirectly confirm cluster analysis
21 results, the discrepancies reported are not surprising, given that these analyses compare different
22 subgroups. For example, we did not find statistically significant differences in nasal polyps
23 frequency between the clusters, whereas, logistic regression analysis identified nasal polyposis as
24 independent predictor of super-response (ER0/OCS0), as expected.⁹ This means that the
25 proportion of patients with nasal polyps is greater among responders than non-responders within
26 each cluster, but the difference in nasal polyp frequency among the clusters is not statistically
27 significant. Therefore, this variable, although useful as independent predictor of response, cannot
28 be used to differentiate clusters from each other.

29 In summary, we identified 4 differential benralizumab responsiveness clusters in severe
30 eosinophilic asthma, according to inflammatory biomarker expression and clinical variables.
31 These clusters might reflect differing inflammatory pathways, but this needs to be shown
32 definitively.

Given the multifactorial nature of clinical responses, machine learning approaches would be preferable to classic analysis methods. The use of Federated databased or Registries would be desirable in order to obtain the large amount of data necessary to enable such approaches.³²

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Conflicts of interest

We declare that we have no conflicts of interest.

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Figure legend

Figure. 1. Benralizumab responsiveness of the clusters. The y-axis reports the percentage of patients who responded and who did not respond according to the 4 criteria used: A, elimination of exacerbations (ER=0); B, elimination of exacerbation and OCS (ER=0, OCS=0); C, elimination of exacerbation and OCS, with ACT score ≥ 20 (ER=0, OCS=0, ACT ≥ 20); D, elimination of exacerbation and OCS, with ACT score ≥ 20 and FEV1 $\geq 80\%$ (ER=0, OCS=0, ACT ≥ 20 , FEV1 ≥ 80). The number of patients for each subgroup (responders/non-responders) are reported within the columns.

1 Table 1. Clinical characteristics of the patients (n=160) prior to Benralizumab.

	Patients
Mean age $\pm$ SD, year	53.8 $\pm$ 13.9
Female, n (%)	109 (68.1)
Mean body weight $\pm$ SD, Kg	71.7 $\pm$ 15.9
Mean height $\pm$ SD, cm	163.7 $\pm$ 9.4
Mean body mass index $\pm$ SD, Kg/m ²	26.6 $\pm$ 5.1
Mean age asthma diagnosis $\pm$ SD, year	33.5 $\pm$ 14.9
Mean disease length $\pm$ SD, year	20.3 $\pm$ 14.3
Atopy (SPT positive for perennial/seasonal allergens), n (%)	96 (60)
Relatives with asthma, n (%)	69 (43.1)
Relatives with atopy, n (%)	76 (47.5)
Smoking history	
- Current smoker, n (%)	11 (6.9)
- Ex smokers, n (%)	30 (18.7)
- Never smoker, n (%)	119 (74.4)
Peripheral blood eosinophil count (cells/ μ L), median (IQR)	690 (460, 975)
Total serum IgE level (kU/L), median (IQR)	134 (57, 368)
FeNO 50 (ppb), median (IQR)	45 (24.7, 62.2)
Pulmonary function tests	
- Mean FVC $\pm$ SD, L	2.75 $\pm$ 1
- Mean FVC $\pm$ SD, % predicted	81.1 $\pm$ 20.4
- Mean pre-bronchodilator FEV ₁ $\pm$ SD, L	1.83 $\pm$ 0.8
- Mean pre-bronchodilator FEV ₁ $\pm$ SD, % predicted	65.6 $\pm$ 21.1
- Mean reversibility in FEV ₁ , % (range)	12 (0 to 54)
[- Patients with reversibility, n (%)]	84 (52.5)
- Mean FEV ₁ /FVC ratio $\pm$ SD, %	66 $\pm$ 13.2
- Mean FEF 25-75 $\pm$ SD, % predicted	41.6 $\pm$ 23.4
Exacerbations in year prior to benralizumab, n (mean $\pm$ SD)	796 (5 $\pm$ 3.4)
- emergency room admission, n (%)	73 (9.2)
- hospitalization, n (%)	36 (4.5)
- Intensive care unit, n (%)	0 (0)

Drug use, number of patients	
- ICS/LABA, n (%)	160 (100)
- LAMA, n (%)	111 (69.4)
- OCS (steroid-dependent), n (%)	95 (59.4)
<i>[- Prednisone eq. median dose in the previous 6 months (IQR), mg]</i>	5 (0, 12.5)
- Leukotriene receptor antagonist, n (%)	76 (47.5)
- Azithromycin, n (%)	21 (13.1)
- Theophylline, n (%)	6 (3.7)
Comorbidities, number of patients	
- Nasal polyposis, n (%)	79 (49.4%)
- EGPA, n (%)	12 (7.5)
- Eosinophilic pneumonia, n (%)	5 (3.1)
- Atopic dermatitis, n (%)	17 (10.6)
- Bronchiectasis, n (%)	35 (21.9)
- Gastroesophageal reflux disease, n (%)	75 (46.9)
- Osteoporosis, n (%)	39 (24.4)
- Anxiety, n (%)	24 (15)
- ASA-sensibility, n (%)	20 (12.5)
- OSAS, n (%)	13 (8.1)
- Urticaria, n (%)	8 (5)
- Depression, n (%)	7 (4.4)
Previous monoclonal antibody treatment, n (%)	8 (5)
- omalizumab, n (%)	5 (3.1)
- mepolizumab, n (%)	5 (3.1)
SNOT 22, mean $\pm$ SD	49.4 $\pm$ 19.7
ACT score at baseline, mean $\pm$ SD	13.6 $\pm$ 4.1
- Patients with ACT $\geq$ 20, n (%)	11 (6.9)
AQLQ score at baseline, mean $\pm$ SD	3.4 $\pm$ 1.1

1

2 SD, standard deviation; IQR, interquartile range; SPT, skin prick test; FeNO, fractional exhaled
3 nitric oxide; FVC, forced vital capacity; FEV₁ = forced expiratory volume in 1 second; FEF 25-75,
4 forced expiratory flow at 25-75% of FVC; PEF, peak expiratory flow; OCS, oral corticosteroids;
5 ICS, inhaled corticosteroids; LABA, long-acting beta2-adrenergic agonist; LAMA, long-acting

- 1 muscarinic antagonist; EGPA, Eosinophilic granulomatosis with polyangiitis; OSAS, Obstructive
2 Sleep Apnea Syndrome; ASA, acetylsalicylic acid; SNOT-22, sino-nasal outcome test; ACT,
3 asthma control test; AQLQ, asthma quality of life questionnaire.

4

Table 2. Outcomes after 6 and 12 months of treatment.

	Baseline	T6	T12	<i>P</i>
Exacerbations, mean±SD	5.0 ± 3.4	0.3 ± 0.7	0.2 ± 0.5	<0.01
Patients (n)	160	134	49	
OCS (prednisone eq.), mg/day, Median(IQR)	5.0 (0.0-12.5)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	<0.01
Patients on OCS (n)*	95/160	24/126	4/47	
Pre-bronchodilator FEV ₁				<0.01
- L, mean±SD	1.83±0.8	2.3±0.8	2.25±0.7	
- %, mean±SD	65.6±21.1	79.3±21.5	76.3±17.8	
Patients (n)	160	103	44	<0.01
ACT, mean±SD	13.6±4.1	20.8±4.1	21.6±3.3	
Patients (n)	160	118	49	
AQLQ, mean±SD	3.4±1.1	5.6±1.1	5.9±1.0	<0.01
Patients (n)	160	82	35	
SNOT 22, mean±SD	49.4±19.7	34.2±20.5	23.5±16.9	<i>NS</i>
Patients (n)	70	63	8	
FeNO (ppb), median (IQR)	45 (24.5, 62.5)	25.5 (15.0, 41.0)	25.0 (18.0, 65.0)	<0.01
Patients (n)	80	46	30	
Eosinophils (cells/μL), median (IQR)	0.69 (0.46, 0.97)	0 (0, 0)	0 (0, 0)	<0.01

Patients (n)	160	115	44	
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SD, standard deviation; IQR, interquartile range; OCS, oral corticosteroids; FEV₁ = forced expiratory volume in 1 second; ACT, asthma control test; AQLQ, asthma quality of life questionnaire; SNOT-22, sino-nasal outcome test; FeNO, fractional exhaled nitric oxide. To compare the 3 measurements in T0, T6 and T12, the Friedman test was used. *numerator: patients for whom the data is available; denominator: patients observed at each specific time-point.

Table 3. Baseline characteristics of the clusters.

Cluster	1 (n=23)	2 (n=19)	3 (n=35)	4 (n=28)	P
Female, (%)	73.9	68.4	60.0	67.9	N.S.
Mean age $\pm$ SD, year	57.6 $\pm$ 9.9	34.9 $\pm$ 8.5	55.1 $\pm$ 12.2	62 $\pm$ 8.7	<0.01
Mean age at onset $\pm$ SD, year	46.3 $\pm$ 8.4	23.8 $\pm$ 10	26.9 $\pm$ 10.3	44.9 $\pm$ 11.9	<0.01
Mean disease length $\pm$ SD, year	11.3 $\pm$ 6.8	11.1 $\pm$ 9.6	28.3 $\pm$ 11.5	17 $\pm$ 9.8	<0.01
Mean body mass index $\pm$ SD, Kg/m ²	26.8 $\pm$ 5.6	28.3 $\pm$ 6.8	26.3 $\pm$ 4.2	26.3 $\pm$ 5.1	N.S.
Relatives with atopy, n (%)	43.5	47.4	85.7	25.0	<0.05
SPT positive (perennial/seasonal allergens)	100	78.9	97.1	7.1	<0.05
Smoking history					N.S.
- Current smoker, n (%)	8.7	0	2.9	0	
- Ex smokers, n (%)	21.7	21.1	25.7	25	
- Never smoker, n (%)	69.6	78.9	71.4	75.0	
Peripheral blood eosinophil count (cells/ μ L), median (IQR)	470 (390, 788)	990 (830, 2100)	700 (540, 900)	745 (700, 840)	<0.05
Median FeNO 50, ppb $\pm$ IQR	43.6 (35, 55)	55 (35.4, 78)	33.5 (14, 59)	47.5 (42, 68)	N.S.
Total serum IgE level (kU/L), median (IQR)	181	225	231	68	<0.01

	(93, 359)	(131, 990)	(185, 457)	(50, 85)	
Mean pre-bronchodilator FEV ₁ % predicted $\pm$ SD	55.7 $\pm$ 17.9	79.3 $\pm$ 17.8	54.1 $\pm$ 20	62.2 $\pm$ 15.5	<0.01
Mean exacerbation number in the year prior to benralizumab, $\pm$ SD	4.0 $\pm$ 2.1	3.6 $\pm$ 1.5	8.4 $\pm$ 3.9	4.1 $\pm$ 2.6	<0.01
Drug use in the 6 months prior to benralizumab,					
- LAMA, patients %	60.9	47.4	82.9	82.1	<i>N.S.</i>
- OCS, patients %	43.5	52.6	71.4	64.3	<i>N.S.</i>
- Mean prednisone eq. dose $\pm$ SD	5.9 $\pm$ 8.8	5.5 $\pm$ 6.9	11.1 $\pm$ 10.4	7.21 $\pm$ 9.1	<i>N.S.</i>
- Azithromycin, %	13	0	22.9	21.4	<0.05
Previous allergen immunotherapy – AIT, patients (%)	30.4	15.8	20	17.9	<i>N.S.</i>
Comorbidities, number of patients					
- Nasal polyposis (%)	34.8	47.4	65.7	60.7	<i>N.S.</i>
- Bronchiectasis (%)	17.4	21.1	42.9	17.9	<i>N.S.</i>
SNOT 22, mean $\pm$ SD	44.3 $\pm$ 18.6	50.9 $\pm$ 20.7	55.1 $\pm$ 21.8	42.38 $\pm$ 17.6	<i>N.S.</i>
ACT score at baseline, mean $\pm$ SD	12.8 $\pm$ 4.9	14.3 $\pm$ 4.7	12.8 $\pm$ 3.9	13.8 $\pm$ 3.4	<i>N.S.</i>
AQLQ score at baseline, mean $\pm$ SD	3.6 $\pm$ 0.9	3.8 $\pm$ 0.7	3.3 $\pm$ 1.2	3.7 $\pm$ 1.2	<i>N.S.</i>

SD, standard deviation; IQR, interquartile range; SPT, skin prick test; FeNO, fractional exhaled nitric oxide; FVC, forced vital capacity; FEV₁ = forced expiratory volume in 1 second; OCS, oral corticosteroids; LAMA, long-acting muscarinic antagonist; SNOT-22, sino-nasal outcome test;

ACT, asthma control test; AQLQ, asthma quality of life questionnaire.

Table 4. Baseline characteristics of superresponders (no OCS use and no exacerbations during the first 6 months after benralizumab; ER0=/OCS=0) and non-superresponders after 6-month of benralizumab treatment.

	Non-Super Responders (N=45)	Super-responders (N=84)	P
Mean age $\pm$ SD, year	54.2 $\pm$ 13.6	53.0 $\pm$ 14.0	<i>NS</i>
Female, n (%)	28 (62.2)	49 (70.2)	<i>NS</i>
Mean body weight $\pm$ SD, Kg	73.6 $\pm$ 17.6	70.7 $\pm$ 15.1	<i>NS</i>
Mean height $\pm$ SD, cm	163.6 $\pm$ 10.0	164.3 $\pm$ 9.4	<i>NS</i>
Mean body mass index $\pm$ SD, Kg/m ²	27.1 $\pm$ 4.8	25.9 $\pm$ 5.6	<i>NS</i>
Mean age at onset $\pm$ SD, year	37.7 $\pm$ 13.7	34.1 $\pm$ 14.2	<i>NS</i>
Mean disease length $\pm$ SD, year	16.3 $\pm$ 10.1	19.0 $\pm$ 12.1	<i>NS</i>
Atopy n (%)	19 (42.2)	42 (50.0)	<i>NS</i>

Smoking history			
- Current smoker, n (%)	2(4.4)	2(2.4)	NS
- Ex smokers, n (%)	13 (28.9)	14 (16.7)	
- Never smoker, n (%)	30 (66.7)	68 (81.0)	
Median Peripheral blood eosinophil count - cells/ μ L (IQR)	680 (470, 920)	747 (495, 1052)	NS
Median total serum IgE level, kU/L (IQR)	114.0 (41-404)	132.5 (66-472.5)	NS
- Mean pre-bronchodilator FEV ₁ $\pm$ SD, L	1.7 $\pm$ 0.7	1.9 $\pm$ 0.8	NS
- Mean pre-bronchodilator FEV ₁ $\pm$ SD, %predicted	60.8 $\pm$ 20.7	60.9 $\pm$ 20.6	NS
- Median FeNO 50, ppb (IQR)	47.3 (23-59)	49.0 ; (35-78)	NS
Exacerbation rate in year prior to benralizumab, n (mean $\pm$ SD)	5.1 $\pm$ 4.2	5.0 $\pm$ 2.9	NS
Drug use, number of patients			
- LAMA, n (%)	36(80.0)	58(69.0)	NS
- OCS, n (%)	33 (73.3)	50 (59.5)	0.01
- Prednisone eq. dose in the previous 6 months, median(IQR)	5 (0, 13)	5 (0, 13)	NS
- Leukotriene receptor antagonist, n (%)	26 (57.8)	39 (46.4)	0.005

- Azithromycin, n (%)	13 (28.9)	8 (9.5)	<i>NS</i>
- <i>Salbutamol daily puffs in the month before benralizumab (mean ± SD)</i>	<i>2.7 ± 2.5</i>	<i>2.2 ± 2</i>	<i>NS</i>
- Immunotherapy, n (%)	7 (15.6)	24 (28.6)	<i>NS</i>
Comorbidities, number of patients			
- Nasal polyposis, n (%)	18 (42.9)	50 (64.9)	0.020
- Bronchiectasis, n (%)	13 (28.9)	16 (19.0)	<i>NS</i>
SNOT 22, mean ± SD	49.4 ± 25	50.3 ± 16.9	<i>NS</i>
ACT score at baseline, mean ± SD	13.1 ± 4.5	13.8 ± 3.9	<i>NS</i>
AQLQ score at baseline, mean ± SD	3.5 ± 0.8	3.2 ± 1.3	<i>NS</i>

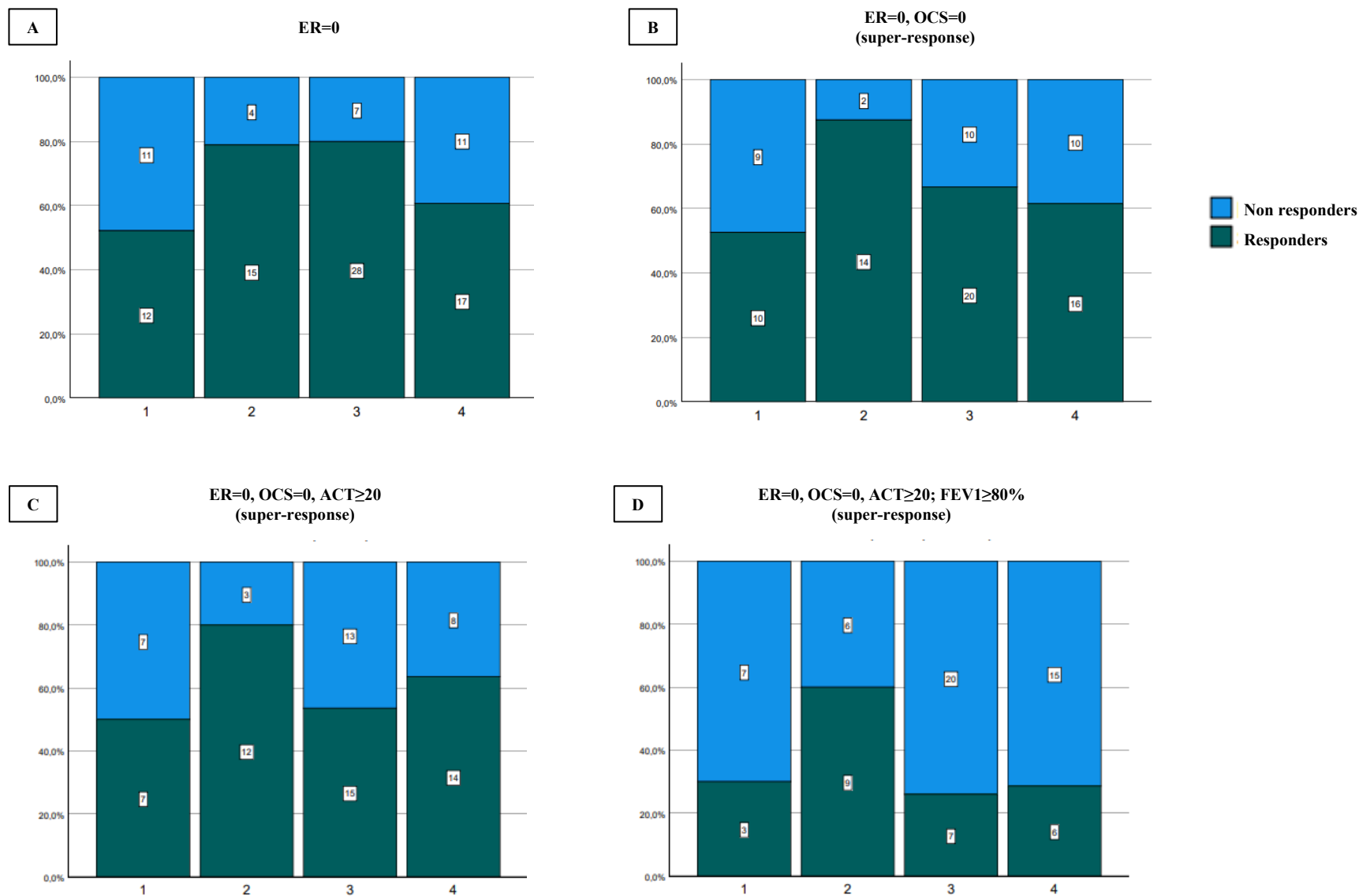
SD, standard deviation; IQR, interquartile range; SPT, skin prick test; FeNO, fractional exhaled nitric oxide; FVC, forced vital capacity; FEV₁ = forced expiratory volume in 1 second; OCS, oral corticosteroids; LAMA, long-acting muscarinic antagonist; SNOT-22, sino-nasal outcome test; ACT, asthma control test; AQLQ, asthma quality of life questionnaire.

Table 5. Logistic regression predicting chance of super-response (ER0/OCS0: no OCS use and no exacerbations during the first 6 months after benralizumab).

Variable	Code	β	Adj. OR	95%CI	<i>P</i> -value
Patient's age		0.039	1.04	1.01; 1.07	0.001
Age at disease onset		-0.44	0.96	0.92; 0.99	0.049
CRwNP	0: No (ref.) / 1: Yes	0.858	2.36	1.07, 5.21	0.034
Azitromycin use	0: No (ref.) / 1: Yes	-1.818	0.16	0.05; 0.52	0.002

CRwNP, chronic rhino-sinusitis with nasal polyps.

Figure 1. Responsiveness to benralizumab by cluster. cea 14026_f1.pptx

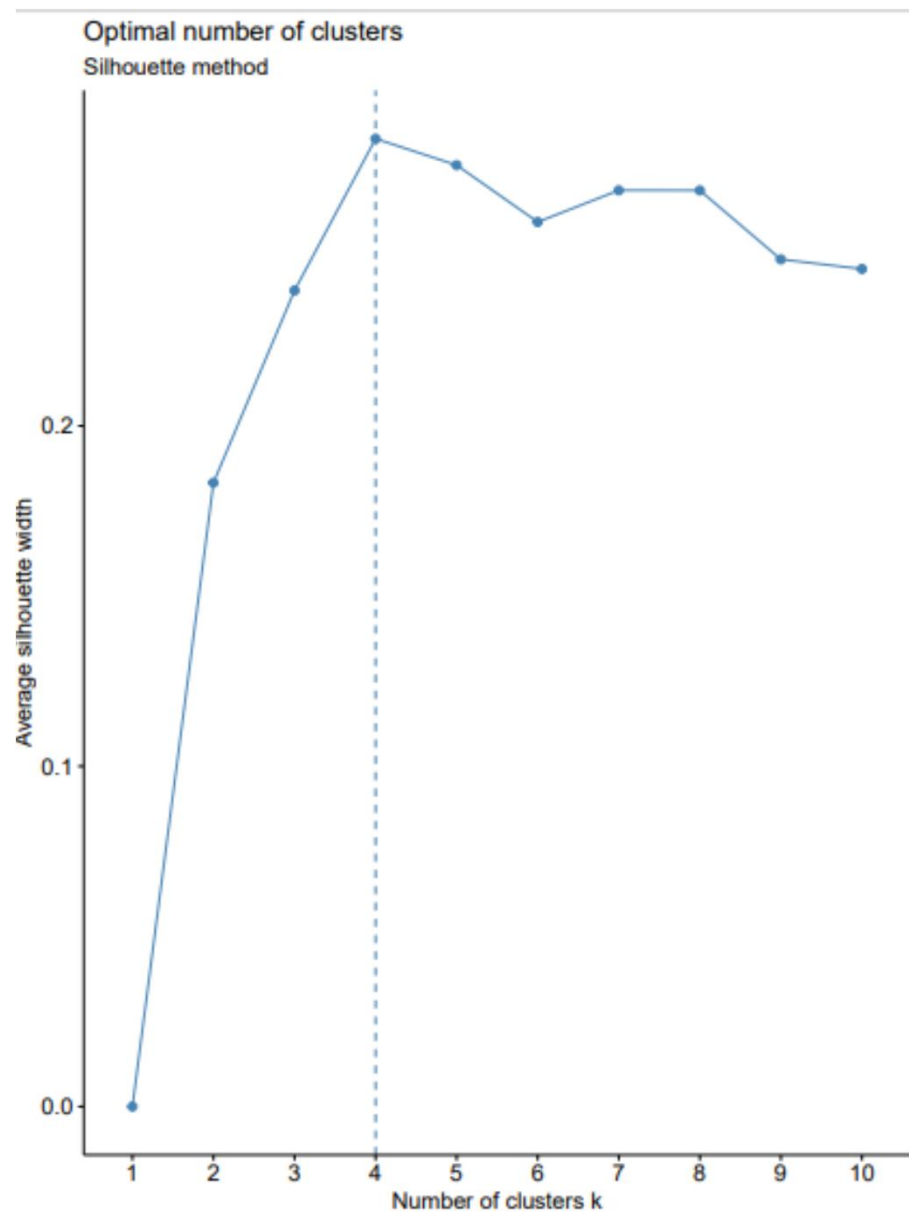


Cluster 1: less eosinophilic, allergic (eosinophils ↓, IgE ↑), older, low nasal polyps frequency, impaired lung function.

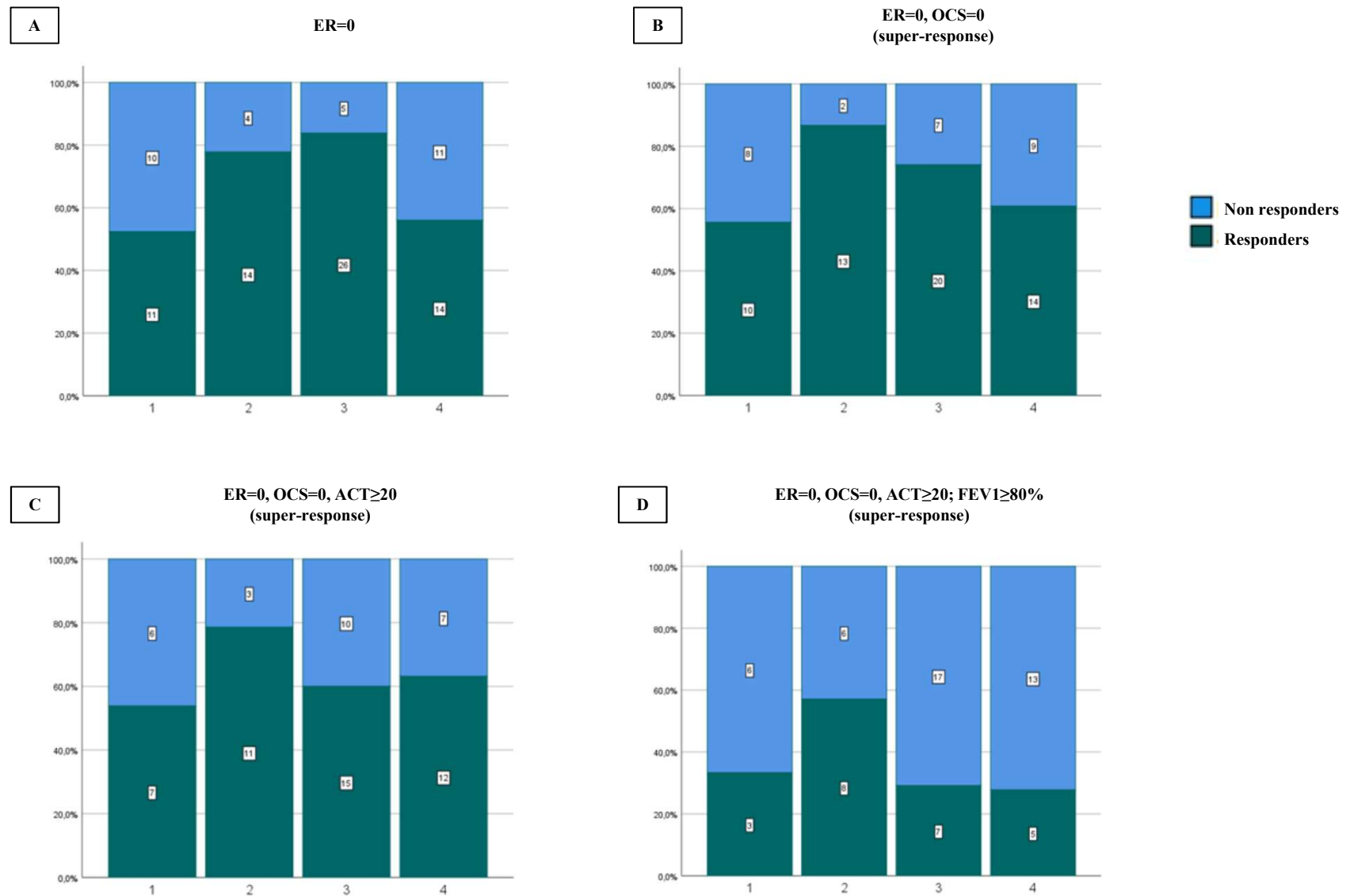
Cluster 2: type-2 biomarkers high (eosinophils ↑, IgE ↑), young, preserved lung function.

Cluster 3: type-2 biomarkers high (eosinophils ↑, IgE ↑), older, exacerbating, nasal polyps, bronchiectasis, impaired lung function.

Cluster 4: eosinophilic, non-allergic (eosinophils ↑, IgE ↓), older, nasal polyps, impaired lung function.



S-Figure 2. Responsiveness to benralizumab by cluster in the subgroup of patients without eosinophilic comorbidities (EGPA, eosinophilic pneumoniae).



Cluster 1: less eosinophilic, allergic (eosinophils ↓, IgE ↑), older, low nasal polyps frequency, impaired lung function.

Cluster 2: type-2 biomarkers high (eosinophils ↑, IgE ↑), young, preserved lung function.

Cluster 3: type-2 biomarkers high (eosinophils ↑, IgE ↑), older, exacerbating, nasal polyps, bronchiectasis, impaired lung function.

Cluster 4: eosinophilic, non-allergic (eosinophils ↑, IgE ↓), older, nasal polyps, impaired lung function.

Supplements

FIGURE LEGENDS

Supplementary-Figure. 1. Silhouette plot to determine the optimal number of clusters.

The silhouette values for each cluster are displayed as the average silhouette width (Y axis), which is the mean of the silhouettes for all items assigned to that cluster. Well clustered items have a value close to 1, poorly clustered items have a value close to -1. The total average silhouette width is the average of the average silhouette widths of the different clusters. The optimal number of clusters is obtained for the maximum of the total silhouette width (X axis).

Supplementary-Figure. 2. Benralizumab responsiveness of the clusters.

The y-axis reports the percentage of patients who responded and who did not respond according to the 4 criteria used: A, elimination of exacerbations (ER=0); B, elimination of exacerbation and OCS (ER=0, OCS=0); C, elimination of exacerbation and OCS, with ACT score ≥ 20 (ER=0, OCS=0, ACT ≥ 20); D, elimination of exacerbation and OCS, with ACT score ≥ 20 and FEV1 $\geq 80\%$ (ER=0, OCS=0, ACT ≥ 20 , FEV1 ≥ 80). The number of patients for each subgroup (responders/non-responders) are reported within the columns.

This analysis was performed in SEA patients after the exclusion of patients with EGPA and eosinophilic pneumonia.