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Basophil activation test for *Staphylococcus aureus* enterotoxins in severe asthmatic patients

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Abstract

Background: Several studies have shown an association between severe asthma and serum immunoglobulins E (IgE) against *Staphylococcus aureus* enterotoxins (SEs). SEs—the prototypes being types A (SEA), B (SEB) and toxic shock syndrome toxin 1 (TSST-1)—can induce both polyclonal and specific IgE responses.

Objective: The aim of the study was to evaluate the ability of SEs to induce basophil activation in severe asthmatic patients using the basophil activation test (BAT).

Methods: 57 severe asthmatic patients were enrolled. BAT in response to SEA, SEB and TSST-1 was performed in all patients, while serum IgE to SEA, SEB and SEC was available in 49 patients. BAT was considered positive when CD203c+ basophils to SEs were $\geq 5\%$, and the stimulation index (SI, ratio between % of CD203c+ basophils to SEs and to negative control) was >2 . Two threshold values (>0.1 kU/L and >0.35 kU/L, respectively) were used to assess serum SEsIgE.

Results: 36.8% of severe asthmatic patients had a BAT positive for at least one SE (BAT SEs+). Serum SEsIgE >0.35 kU/L (SEs IgE+) was associated with BAT SEs positivity. Among patients with negative skin prick test, 35% were BAT SEs+, 30% SEs IgE+, 55% BAT or IgE- SEs+. A negative correlation between SI of BAT to SEs and both clinical (ACT score) and functional parameters was observed, together with a positive correlation of BAT with asthma exacerbations.

Conclusions: The positivity of BAT for SEs in a subgroup of severe asthmatic patients further supports the pathogenic role of *Staphylococcus aureus* in severe asthma.

KEYWORDS

basophil activation test, disability, outcome, severe asthma, *Staphylococcus aureus* enterotoxins

1 | INTRODUCTION

Severe uncontrolled asthma is a heterogeneous disease characterized by the persistence of the symptoms, history of exacerbations, and impaired lung function, despite the use of high doses

of anti-asthma drugs recommended by GINA guidelines.¹ Several clinical studies have reported an association between the sensitization to *Staphylococcus aureus* enterotoxins (SEs) and severe asthma.^{2–6} In particular, a recent longitudinal study has shown that SE sensitization is associated with an increased risk of developing

severe asthma and disease exacerbations.⁷ However, the role played by SEs in the pathogenesis of severe asthma has not yet been fully clarified.

SEs—the prototypes being types A (SEA), B (SEB) through E, and toxic shock syndrome toxin 1 (TSST-1)—are a small group of globular proteins with a molecular weight of about 22 kDa that can act as T-cell superantigens, and therefore can strongly trigger T-cell proliferation and activation. Activated T cells can release interleukins (ILs)—such as IL-4, IL-5, IL-13—as well as eotaxin, which can lead to eosinophilic inflammation and tissue polyclonal IgE response, usually associated with an increase in total serum IgE.⁸ In addition to their superantigenic activity, SEs can act as allergens, thereby inducing the production of specific IgE.⁹ It is unclear whether the polyclonal IgEs produced in response to SEs are functional, and therefore can bind to the Fcε receptors I expressed by mast cells and basophils and activate them. It has been speculated that these IgEs can be non-functional, or may even prevent the bridging of IgE molecules by competing for the IgE-binding sites of an allergen on the mast cell surface.¹⁰ A few in vitro studies have investigated the role played by IgEs towards SEs. Leung et al¹¹ showed that the basophils isolated from the peripheral blood of patients with atopic dermatitis were able to release histamine upon exposure to the specific SEs they were sensitized to. Zhang et al¹⁰ demonstrated that mucosal IgEs to SEs from nasal polyps were capable of activating mast cells, regardless of their presence in the serum. In asthmatic patients, an in vitro study has shown that *Staphylococcus aureus* can induce histamine release from human basophils and lung mast cells.¹² However, the role of SEs in inducing mast cell and/or basophil activation in asthmatic patients has never been investigated.

The basophil activation test (BAT) is an assay that can be considered an in vitro surrogate of an in vivo acute allergic reaction. Unlike the measurement of serum specific IgEs, BAT evaluates only functional IgEs. In sensitized patients, the stimulation of peripheral blood basophils with a specific allergen induces or up-regulates the expression of activation markers on the cellular surface, which can be measured by flow cytometry. BAT is a diagnostic test validated in many IgE-mediated responses, including drug, food, venom and pollen allergies. It allows to monitor the clinical effects of either immunotherapy or anti-IgE treatment (13), as well as to assess the severity of the allergic reactions.^{13,14}

Based on the above considerations, in the present study we used BAT, with the aim of evaluating the ability of SEs to induce basophil activation in severe asthmatic patients.

2 | METHODS

2.1 | Study population

We prospectively enrolled 57 patients with severe asthma from the outpatient service of the Pulmonology Unit of the University of Campania 'Luigi Vanvitelli', Naples, Italy. All patients had their

diagnosis of asthma confirmed, comorbidities assessed and treated, inhalation technique and adherence verified, and therapy optimized.

The inclusion criteria were as follows: (a) severe uncontrolled asthma according to ERS/ATS guidelines¹; (b) age > 18; (c) asthma therapy according to GINA guidelines, step 5, for at least three months.

The exclusion criteria were as follows: (a) recent respiratory infections during the last 4 weeks; (b) therapy with antibiotics and/or systemic corticosteroids over the last 4 weeks; (c) biological therapy for asthma; (d) other concomitant lung diseases (COPD, lung cancer, tuberculosis, interstitial lung diseases); (e) conditions associated with unusually high serum IgE levels or eosinophils (eg allergic bronchopulmonary aspergillosis, eosinophilic granulomatosis with polyangiitis, eosinophilic pneumonia, hyper-IgE syndrome); (f) systemic diseases (malignancy, immunodeficiency, connectivitis).

2.2 | Baseline characteristics

All patients underwent the following:

Anamnesis and complete medical examination. In particular, we evaluated these characteristics: age at the onset of asthma; smoking status, defined as never, current or former smoker; history of acetylsalicylic acid intolerance or other drug reactions; body mass index (BMI), calculated as weight/height squared; comorbidities, such as chronic rhinosinusitis (CRS) with or without nasal polyps (CRSwNP and CRSsNP, respectively), gastroesophageal reflux disease (GERD) and atopic dermatitis; clinically significant exacerbations over the previous year, defined as asthma deterioration requiring the use of systemic corticosteroids for more than 3 days and/or hospital admission.

Skin prick tests, performed using standard series of inhaled allergens (Lofarma).

Asthma control test (ACT), a validated five-item questionnaire for assessing asthma control. Each item is scored on a five-point Likert scale from 1 to 5 (1 = worst; 5 = best), with a total score of <5, 5–19 and 20–25 points describing uncontrolled, partially controlled and well-controlled asthma, respectively.

Sinonasal Outcome Test (SNOT 22) questionnaire. It rates 22 different symptoms, from 0 (no problem) to 5 (problem as bad as it can be) related to rhinological, ear, facial, general, physical and psychological domains.¹⁵

Spirometry (model 'Quark PTF', COSMED) before (pre-BD) and after (post-BD) bronchodilator (salbutamol 400 mcg), performed according to the guidelines of the American Thoracic Society/European Respiratory Society (ATS/ERS)¹⁶; Forced Expiratory Volume in one second (FEV₁), Forced Vital Capacity (FVC), and FEV₁/FVC ratio were recorded, and the best of three forced manoeuvres was recorded. The results were expressed as absolute values, and as a percentage of the predicted values referred to 1993 European Respiratory Society (ERS) reference values.

Rhinolaryngoscopy and/or paranasal sinus computed tomography was performed, in order to validate a diagnosis of CRSsNP or CRSwNP; nasal polyps were also deemed positive if the subject had a history of NP surgery.

Fractional Exhaled Nitric oxide (FeNO) was analysed using an electrochemical analyzer (HypairFeNOMedisoftExp'air, 2010), according to the ATS/ERS recommendations for the online measurement of exhaled NO in adults.^{17,18}

Peripheral blood eosinophil counts were measured using an automated haematology analyzer.

Serum total IgE measurement (ImmunoCAP system; Phadia).

2.3 | Serum IgE to SEs

Specific IgE to SEA, SEB, and SEC was measured through the ImmunoCAP system (Phadia). Two threshold values were used to evaluate the sensitization to SEs >0.1 and >0.35 kU/L, respectively—according to literature data.¹⁹ In addition, also ratios between serum-specific enterotoxin IgE/total IgE were assessed.

2.4 | Basophil activation test for SEs

Basophil activation test (BAT) in response to SEA, SEB and TSST was performed by flow cytometry (FC500, Beckman Coulter, with CXP Analysis software). The Allergenicity Kit (Beckman Coulter) was used for the quantification of the basophil CD203c expression, according to the instructions supplied by the manufacturer. CD203c is an enzyme that breaks phosphodiester and phosphosulphate bonds, hydrolytically removing 5-nucleotides in succession from the 3'-hydroxytermini of the oligonucleotides. It is exclusively and constitutively expressed at low levels on the surface of basophils and mast cells, and its expression increases with cell activation. In order to define the best stimulating concentrations of SEs, we performed some experiments of limiting dilution. According to Leung et al, our results suggested that the concentration of SEs able to elicit the best dose-response in the sensitized basophils was 10 $\mu\text{g/mL}$. Briefly, 100 μL aliquots of the patient's whole blood anticoagulated with EDTA were prepared for in vitro stimulation. 20 μL of phosphate-buffered saline (PBS) as a negative control, or 20 μL of anti-IgE as a positive control, or 5 μL of SEA, SEB, and TSST extracts (10 $\mu\text{g/mL}$, Sigma), were added to the blood (processed as described above), and the mixture was incubated for 15 minutes at 37°C.¹¹ Basophils were identified by their characteristic forward and side scatter, and also on the basis of CRTH2 expression, in the absence of CD3. Results were considered positive when the absolute percentage of basophils expressing CD203c after contact with SEs was $\geq 5\%$, and the stimulation index (SI), calculated as the ratio between the percentage of basophils expressing CD203c upon SEs stimulation and the percentage of basophils expressing CD203c in the negative control, was >2 .²⁰ We also calculated the ratio between the percentage of CD203c+

basophils after stimulation with SEs, as well as the percentage of CD203c+ basophils after stimulation with anti-IgE.¹⁴

This observational study was carried out according to the standards of Good Clinical Practice and to the principles of the Declaration of Helsinki. Our trial was approved by the local Ethics Committee (number 752/4-12-2019—Università della Campania 'Luigi Vanvitelli', AORN 'Ospedali dei Colli'), and all enrolled patients gave their written informed consent.

2.5 | Statistical analysis

Data were reported as mean values $\pm$ standard deviation (SD) or median (interquartile range), and compared by using Student's *t* test, analysis of variance (ANOVA) or Mann-Whitney *U* test. Categorical variables were given as frequency with percentage, and associations were evaluated with chi-squared or Fisher's exact test, as appropriate. The Spearman's rank correlation coefficient (ρ) was used to measure the strength of the relationship between variables. Logistic regression was used to estimate multivariate Odds Ratio for BAT positivity. A *P* value of $<.05$ was considered as statistically significant. These analyses were made using STATA software, version 14 (Stata Corp).

3 | RESULTS

The study enrolled 57 patients with severe uncontrolled asthma. BAT to SEs was performed in all patients, while the measurement of serum IgE to SEs was available in 49 patients. Examples of BAT positive for SEs showing the 'gating', 'threshold' and 'analysis' strategy are shown in Figures 1 and 2.

Twenty-one patients (37%) had aBAT positive for at least one SE (BAT SEs+ group): in particular, 12 patients (21%) had a BAT positive for SEA, 15 (26%) for SEB and 13 (23%) for TSST. 36 patients (63%) had a test negative for all SEs (BAT SEs- group). In Table 1, we showed, in subgroups of patients with positive or negative BAT for SEA, SEB and TSST-1, the percentages of activated basophils following the stimulation with the specific enterotoxin, with anti-IgE (positive control) and in the negative control, and the relative ratios. Further, in Figure 3 we showed dot plots of individual SI ratio of BAT responses to SEA, SEB and TSST-1 in positive vs negative patients.

Among the 49 severe asthmatic patients who underwent both BAT and the measurement of serum IgE to SEs, 20 patients (41%) had BAT SEs+, 28 patients (57%) had serum levels of IgE to at least one SE. >0.10 kU/L and 14 patients (29%) had serum levels of IgE to at least one SE >0.35 kU/L.

In comparison with the BAT SEs- group, the BAT SEs+ group included a significantly higher percentage of patients with serum SEs IgE >0.35 kU/L (17% vs 45%, $P = .035$), but not with serum IgE >0.1 kU/L (55% vs 60%, $P = .737$) (Figure 4).

Considering the higher association between BAT SEs+ group and serum IgE to SEs >0.35 kU/L, we analysed the baseline

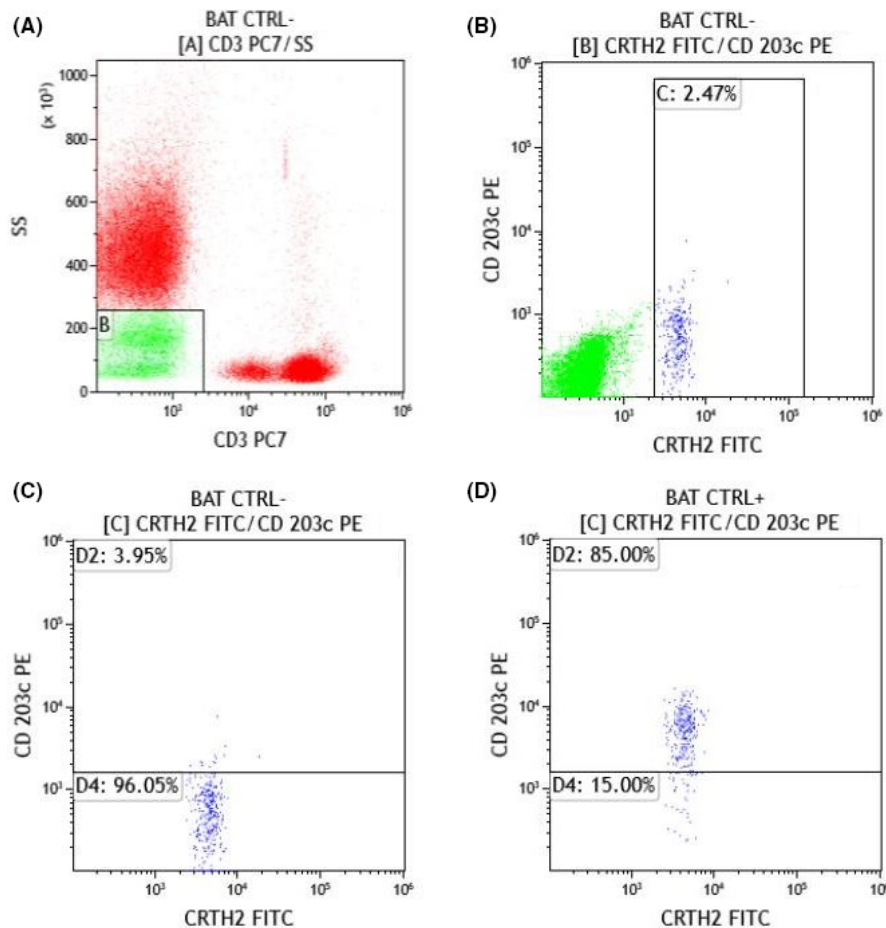


FIGURE 1 A clear example of a BAT positive for SEs illustrating the 'gating' and 'threshold' strategy. Panel A, The 'gating strategy': a gate is open on the lymphomonocytic region in order to exclude neutrophils and eosinophils, by the side scatter characteristics, and lymphocyte T subset, by the positivity to CD3. Panel B, Basophils are characterized by CRTH2 positivity; Panel C and D, 'The threshold strategy': The lowest negative value of the negative control (Panel C) which allows to define the highest value of the positive control (Panel D) and, consequently, the best definition of basophil reactions to SEs, was identified

demographic, clinical and functional characteristics of the asthmatic patients according to the presence of serum IgE to at least one SE >0.35 kU/L (IgE SEs+ group) or serum IgE to all SEs ≤ 0.35 kU/L (IgE SEs- group), and in the BAT SEs+ or BAT SEs- groups (Table 2).

Clinical, functional and laboratory findings were not significantly different between BAT SEs+ and BAT SEs- groups, and between IgE SEs+ and IgE SEs- groups, except for the previously described relationship between BAT and IgE SEs positivity >0.35 kU/L and for the higher level of total serum IgE in the IgE SEs+ group (Table 2).

The subgroup of severe asthmatic patients with both BAT and serum IgE positive to SEs as well as the subgroup of patients positive only to serum IgE to SEs had total serum IgE significantly higher compared to those who were only positive to BAT or negative to both ($P = .024$, $P = .038$, $P = .002$, $P = .002$, respectively) (Figure 5).

In the BAT positive SEA and/or SEB subgroup, the ratio between serum SE-specific IgE/total IgE was higher as compared with that of the BAT negative SEA and/or SEB subgroup, although it did not reach the statistical significant difference (4×10^{-4} [$1 \times 10^{-5} - 2 \times 10^{-3}$] vs 3×10^{-4} [$1 \times 10^{-4} - 9 \times 10^{-4}$], $P = .373$).

In the study group, 24 (42%) severe asthmatic patients, defined as 'non-allergic' on the basis of the negativity of the skin prick test for common inhaled allergens, showed a median value of serum total IgE of 147 kU/L (73-453). In 20 non-allergic patients, the assessment of both BAT and serum IgE to SEs was available. Among them,

we identified seven patients (35%) BAT SEs+, 6 (30%) IgE SEs+, 11 (55%) BAT or IgE SEs+, and 2 (10%) BAT and IgE SEs+.

A significant negative correlation was identified between the stimulation index (SI) of BAT to all SEs and the spirometric parameters $FEV_1\%$, FVC%, and their post-bronchodilator values (Table 3, Figure S1), and between the SI of BAT to SEA or SEB and ACT (Table 3, Figure S2). A significant positive correlation was found between the SI of BAT to SEA or TSST and the history of clinically significant asthma exacerbations in the previous year (Table 3, Figure S3).

In addition to SI, we further investigated the ratio between the percentage of basophils expressing CD203c after SEA, SEB or TSST stimulation, and the percentage of basophils expressing CD203c following anti-IgE stimulation. We observed a significant inverse correlation of this ratio with FEV_1/FVC (Table 3, Figure S4).

4 | DISCUSSION

In the present study, we performed for the first time the BAT for SEs, in order to evaluate the ability of SEs to induce basophil activation in severe asthma. We observed that more than one third of the severe asthmatic patients enrolled in the study showed a positive BAT to at least one SE. This result can have, in our opinion, a clinical relevance. In fact, although the association between severe asthma and

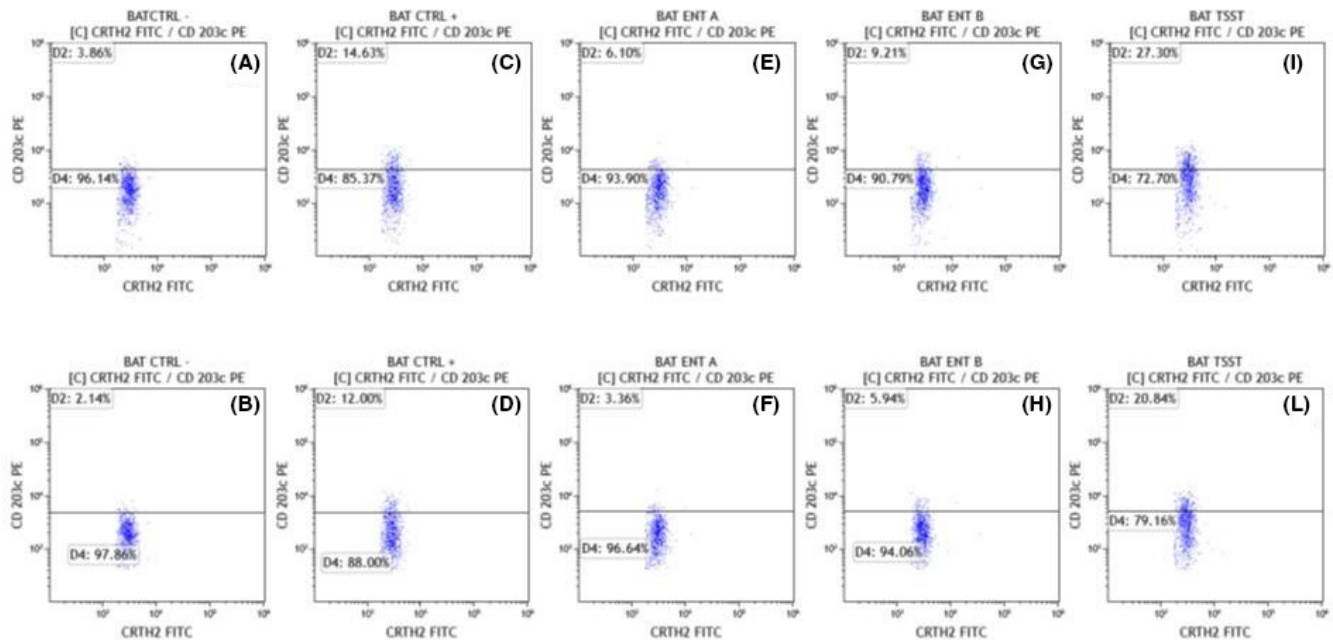


FIGURE 2 The ‘analysis strategy’ in a more difficult example of a BAT positive for SEs. In panel A and in panel B, we demonstrate how a little variation of the threshold can modify the determination of the positive reaction. We always choose the lowest negative control that allows to define the highest positive control. Among A and B, we choose A and subsequently C (positive control), E (ENT A), G (ENT B) and I (TSST-1)

TABLE 1 Percentages of activated basophils in the negative control and following the stimulation with anti-IgE (positive control) and with the specific SE and the relative ratios in positive or negative BAT for SEA, SEB and TSST-1

	BAT SEA		BAT SEB		BAT TSST	
	Positive (n = 12)	Negative (n = 45)	Positive (n = 15)	Negative (n = 42)	Positive (n = 13)	Negative (n = 44)
% basophil activation, median [IQR]						
Negative control	4.3 [1.9-5.8]	4.7 [2.4-8.1]	3.8 [2.2-6]	5 [2.5-8.2]	3.7 [2.4-5.4]	5.1 [2.4-8.1]
Positive control (anti-IgE)	67.6 [42.4-82.3]	38.9 [16.3-58.7]	62.6 [50.8-80.4]	38.1 [16.3-55.2]	62.6 [47.9-77.4]	38.1 [16.2-57.2]
Specific SE	17.4 [11.6-20.1]	5.05 [2.9-8.2]	15.5 [8.3-39.8]	5.5 [2.9-8.1]	16.4 [12.6-38.5]	4.2 [2.9-7.3]
Ratios, median [IQR]						
SE IgE/Negative Control (SI)	4.4 [2.5-6.1]	1.08 [0.78-1.43]	3.02 [2.2-8.5]	1.01 [0.74-1.43]	3.8 [3.2-13.1]	1.06 [0.75-1.43]
SE IgE/Anti-IgE	0.32 [0.17-0.41]	0.13 [0.06-0.33]	0.32 [0.13-0.78]	0.15 [0.09-0.29]	0.26 [0.20-0.91]	0.14 [0.07-0.35]

Abbreviations: SE, *Staphylococcus aureus* enterotoxin; SI, stimulation index.

the presence of serum IgE to SEs has been demonstrated by several studies,²⁻⁶ IgE to staphylococcus enterotoxins could be a result of a polyclonal activation with lack of clinical relevance. The ability of SEs to activate basophils in vivo, demonstrated in a subgroup of severe asthmatic patients through BAT, further supports the role of SEs as triggers of the IgE-mediated immune-inflammatory cascade. It is still questionable whether SEs can be considered as perennial inhalant allergens. *Staphylococcus aureus* frequently colonizes the upper airways, especially anterior nares and nasal polyp tissue, where it can release SEs.²¹ In addition, a recent study has shown that *Staphylococcus aureus* is also a part of the microbiome of house dust mites (HDMs), and SEs could be inhaled through the latter in the role of carriers.²²

Consistently with our data, previous studies have reported that about one third of asthmatic patients with negative skin prick tests had high levels of total serum IgE, thus suggesting a sensitization to unidentified allergens.^{23,24} Bachert et al² showed that sensitization to SEs occurred in 21% of severe asthmatic patients previously considered as non-atopic. Such findings have been recently confirmed by the ‘Identify Project’, which detected the presence of serum IgE to SEA and/or SEB in 23% of 362 severe asthmatic patients with prick tests negative for the standard panel of aeroallergens.²⁵ We found the presence of serum IgE to SEs in 30% of non-allergic patients, but this prevalence increased to 55% when we evaluated the sensitization to SEs using also BAT.

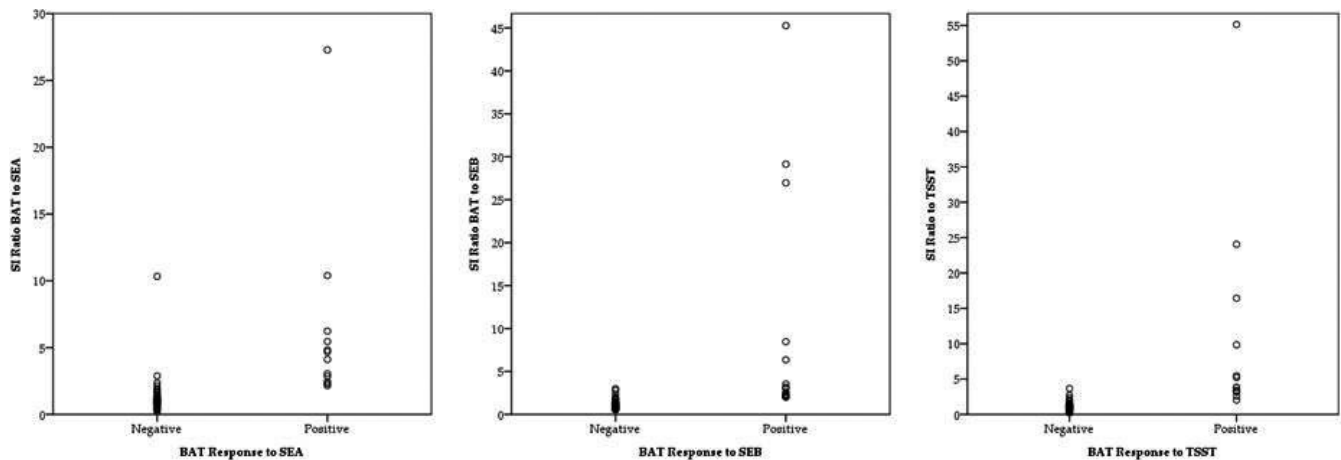


FIGURE 3 Dot plots of individual SI ratios of BAT response to SEA, SEB and TSST-1 for positive vs negative patients

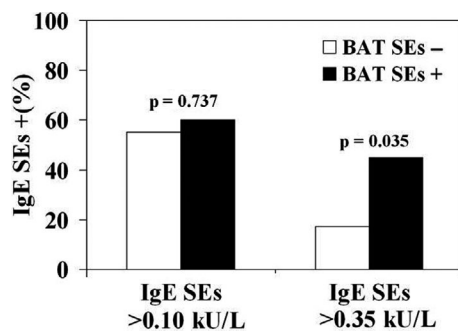


FIGURE 4 A significantly higher percentage of patients with serum SEs IgE >0.35 kU/L, but not with SEs IgE >0.10 kU/L, was detected in BAT SEs+ patients, when compared to the BAT SEs- subgroup. Abbreviations: SEs: *Staphylococcus aureus* enterotoxins; BAT SEs-, Basophil activation test negative for all SEs; BAT SEs+, Basophil activation test positive for at least one SE

Interestingly, in the present study we observed that not all patients with serum IgE to SEs had a positive BAT test. The probability of having a BAT test positive for SEs increased when we considered a threshold of serum IgE to SEs higher than 0.35 kU/L, rather than the lower cut-off of 0.1 kU/L utilized in several other clinical studies. Furthermore, the ratio between serum-specific SE IgE and total IgE was higher in the BAT positive subgroups in comparison with the BAT negative subgroups, although the difference was not statistically different. It has been suggested that the higher IgE-ab fraction correlated with the possibility for basophils to respond to allergen stimulation.⁹ In comparison with the standard measurement of serum IgE, BAT only evaluates functional IgE. In fact, two clones of IgE on sensitized basophil surface able to bind non-overlapping epitopes on allergen can determine basophil activation with consequent increase of CD203C expression detected by flow cytometry in the BAT. Already validated as a sensitive and specific test to diagnose several allergic conditions,¹³ BAT has also been recently shown to be an assay that can discriminate allergic bronchopulmonary aspergillosis from *Aspergillus fumigatus* colonization in cystic fibrosis.²⁶ Moreover, Mirkovic et al²⁷ demonstrated

a correlation between the basophil expression of CD203c after stimulation with *Aspergillus fumigatus* and some indices of negative prognosis in cystic fibrosis patients, such as lower pulmonary function and BMI. Likewise, we observed a relationship between SI of BAT for SEs and several functional and clinical parameters relevant to asthma severity, such as spirometry parameters, ACT and a history of asthma exacerbations, though we did not find any clinical and functional difference between BAT SEs positive and negative subgroups. Furthermore, the severity of airway obstruction was also related to a marker of the severity of allergic reactions, that is, the ratio between the percentages of basophils expressing CD203c after stimulation with SEs and anti-IgE.¹⁴ Differently from the literature evidence that showed an association between SE IgE positivity and several features of severe asthma, such as the need for oral steroid intake, hospitalizations and impaired lung function, we did not observe any relationship between serum levels of IgE to SEs and asthma severity.⁶ In this regard, we suppose that the homogeneity of the sample of severe asthmatic patients enrolled in our study, which did not recruit subjects with more moderate degrees of asthma severity, might have mitigated the above correlation.

In the present study, 40% of patients with BAT positive for at least one SE had undetectable levels of serum IgE to SEs. Several studies have emphasized the role of local IgE production in both allergic and non-allergic asthma,²⁸⁻³⁰ and the presence of mucosal SE IgE has been demonstrated in nasal polyp tissue.¹⁰ A low spillover into the circulation of mucosal allergen-specific IgE could occur in some patients considered as non-atopic.³¹ These levels of serum IgE, although they do not reach the cut off of serum SE IgE detection, can sensitize basophils thus rendering BAT positive. In rhinitic patients lacking both skin prick test positivity and serum specific IgE, BAT showed a good sensitivity in diagnosing local allergic rhinitis, thus suggesting a relationship with a local IgE response.³² In allergic reactions, basophils behave as circulating effector cells similar to tissue mast cells, and BAT could thereby reflect mast cell responsiveness and local IgE production better than serum IgE antibodies.

TABLE 2 Baseline population characteristics

	All (n = 57)	BAT SEs- (n = 36)	BAT SEs+ (n = 21)	P	IgE SEs- (n = 35)	IgE SEs+ (n = 14)	P
Age (y), mean $\pm$ SD	57 $\pm$ 13	58 $\pm$ 9	56 $\pm$ 17	ns	57 $\pm$ 12	56 $\pm$ 14	ns
Male gender, n (%)	8 (14)	4 (11)	4 (19)	ns	4 (11)	3 (21)	ns
Asthma onset (y), mean $\pm$ SD	34 $\pm$ 16	37 $\pm$ 15	30 $\pm$ 18	ns	33 $\pm$ 17	35 $\pm$ 15	ns
BMI (kg/m ²), mean $\pm$ SD	28.3 $\pm$ 4.3	27.8 $\pm$ 4.2	28.5 $\pm$ 3.6	ns	28.1 $\pm$ 4.0	28.5 $\pm$ 3.9	ns
Former smoker, n (%)	30 (53)	18 (50)	12 (57)	ns	17 (49)	10 (71)	ns
Never smoker, n (%)	27 (47)	18 (50)	7 (43)	ns	18 (51)	4 (29)	ns
ACT, mean $\pm$ SD	11.7 $\pm$ 3.8	12.0 $\pm$ 3.9	11.3 $\pm$ 3.7	ns	12.2 $\pm$ 4.0	10.9 $\pm$ 3.0	ns
SNOT-22, mean $\pm$ SD	52 $\pm$ 22	50 $\pm$ 24	54 $\pm$ 18	ns	55 $\pm$ 26	43 $\pm$ 17	ns
Exacerbation/last year, mean $\pm$ SD	6 $\pm$ 5	6 $\pm$ 5	7 $\pm$ 6	ns	6 $\pm$ 5	7 $\pm$ 5	ns
Hospitalization/last year, mean $\pm$ SD	0.9 $\pm$ 1.3	0.6 $\pm$ 1.1	1.6 $\pm$ 1.8	ns	1.1 $\pm$ 1.4	0.7 $\pm$ 1.2	ns
Allergy, n (%)	33 (58)	19 (54)	13 (62)	ns	20 (59)	8 (57)	ns
CRSsNP, n (%)	17 (30)	11 (31)	6 (29)	ns	12 (34)	4 (29)	ns
CRSwNP, n (%)	20 (35)	13 (36)	6 (29)	ns	10 (29)	6 (43)	ns
GERD, n (%)	31 (54)	21 (58)	9 (43)	ns	22 (63)	5 (36)	ns
Atopic dermatitis, n (%)	5 (9)	5 (14)	0 (0)	ns	4 (11)	0 (0)	ns
FeNO (ppb), mean $\pm$ SD	33 $\pm$ 26	34 $\pm$ 29	29 $\pm$ 17	ns	32 $\pm$ 23	27 $\pm$ 33	ns
FEV ₁ (L), mean $\pm$ SD	1.26 $\pm$ 0.53	1.26 $\pm$ 0.48	1.27 $\pm$ 0.64	ns	1.31 $\pm$ 0.59	1.28 $\pm$ 0.39	ns
FEV ₁ (% predicted), mean $\pm$ SD	53 $\pm$ 17	55 $\pm$ 14	51 $\pm$ 21	ns	55 $\pm$ 18	52 $\pm$ 16	ns
FVC (L), mean $\pm$ SD	2.12 $\pm$ 0.74	2.13 $\pm$ 0.73	2.11 $\pm$ 0.79	ns	2.15 $\pm$ 0.78	2.21 $\pm$ 0.61	ns
FVC (% predicted), mean $\pm$ SD	75 $\pm$ 19	78 $\pm$ 19	72 $\pm$ 20	ns	77 $\pm$ 20	75 $\pm$ 21	ns
FEV ₁ /FVC, mean $\pm$ SD	0.60 $\pm$ 0.11	0.60 $\pm$ 0.11	0.59 $\pm$ 0.13	ns	0.60 $\pm$ 0.11	0.59 $\pm$ 0.13	ns
Total serum IgE (kU/L), median (range)	147 (73-453)	123 (53-334)	207 (97-483)	ns	120 (52-232)	513 (299-1150)	<0.001
Eosinophil (cell/mm ³), median (range)	350 (140-670)	385 (210-665)	200 (110-680)	ns	330 (120-680)	515 (110-840)	ns
Eosinophil (%), mean $\pm$ SD	5.4 $\pm$ 4.2	5.7 $\pm$ 3.9	4.9 $\pm$ 4.8	ns	5.1 $\pm$ 4.0	6.6 $\pm$ 5.2	ns
IgE SEA, SEB, SEC >0.1 kU/L, n (%)	28 (57)	16 (55)	12 (60)	ns			
IgE SEA, SEB, SEC >0.35 kU/L, n (%)	14 (29)	5 (17)	9 (45)	0.035			
BAT SEA, SEB or TSST-1+, n (%)	21 (37)			ns	11 (31)	9 (64)	0.035

Abbreviations: ACT, asthma control test; BAT SEs-, Basophil activation test negative for all SEs; BAT SEs+, Basophil activation test positive for at least one SE; BMI, Body mass index; CRSsNP, chronic rhinosinusitis without nasal polyps; CRSwNP, chronic rhinosinusitis with nasal polyps; FeNO, Fractional Exhaled Nitric oxide; FEV₁, Forced Expiratory Volume in one second; FVC, Forced Vital Capacity; IgE SEs-, Serum IgE to all SEs $\leq$ 0.35 kU/L; IgE SEs+, Serum IgE to at least one SE >0.35 kU/L; SEA, *Staphylococcus aureus* enterotoxin A; SEB *Staphylococcus aureus* enterotoxin B; SEC, *Staphylococcus aureus* enterotoxin C; SEs, *Staphylococcus aureus* enterotoxins; SNOT 22, Sinonasal Outcome Test 22; TSST-1, toxic shock syndrome toxin 1.

Among the non-allergic patients enrolled in the study group in which both BAT and serum IgE to SEs were evaluated, we observed that 30% had IgE to at least one SE, and the percentage of patients

sensitized to SEs reached 55% when BAT was also performed. In addition, we demonstrated a relationship between serum levels of IgE to SEA or SEB and total serum IgE, particularly in patients who

also showed a BAT positivity. An important limitation of the present study is that the SEs used to detect serum IgE and to stimulate basophils were not the same. This could be responsible for the discrepancy observed between the two diagnostic procedures in the same patient. Moreover, because of different commercial availabilities, SEC was used to detect only serum IgE, while TSST-1 was used only for BAT.

The clinical consequences of a stimulation with SEs have never been investigated. However, serum levels of IgE to SEs have been

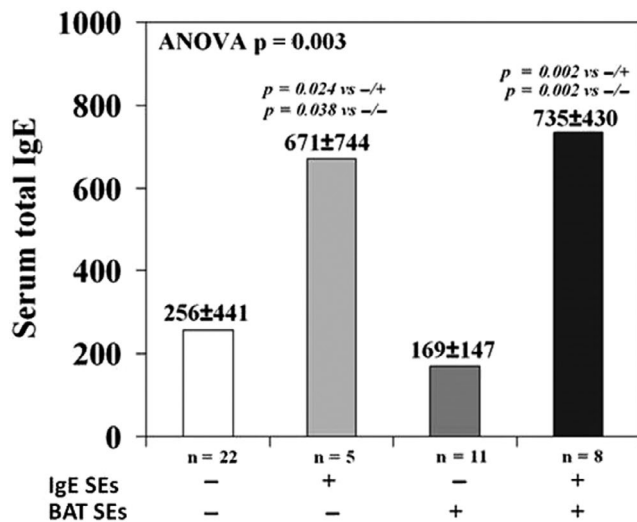


FIGURE 5 Serum total IgE was significantly higher in the subgroups IgE SEs+/BAT SEs- and IgE SEs+/BAT SEs+ compared to those who were positive to BAT only or negative to both. The analysis was performed after natural logarithm transformation of serum total IgE. BAT SEs+, Basophil activation test positive for at least one SE; IgE SEs+, Serum IgE to at least one SE >0.35 kU/L; SEs, *Staphylococcus aureus* enterotoxins

associated with both clinical features of asthma severity and chronic rhinosinusitis, a frequent comorbidity of severe asthma.

The demonstration of the functional properties exhibited by SE-specific IgE in severe asthmatics, as well as the potential role of BAT in detecting mucosal SE-binding IgE, even in non-allergic patients, might have important therapeutic implications. In particular, patients sensitized to SEs could be potential candidates to biological therapies with anti-IgE monoclonal antibodies. Indeed, several studies have shown the efficacy of anti-IgE treatment also in some subjects with intrinsic asthma, who responded to add-on omalizumab therapy, with significant improvements in symptom control, quality of life, lung function, and asthma exacerbation rate.³³⁻³⁵ Furthermore, a recent study has shown the efficacy of omalizumab in non-atopic patients with nasal polyps and asthma comorbidity, most of whom were positive for SE IgE.³⁶ However, these subjects could also be potentially eligible to other, new biological therapies. In fact, it has been recently demonstrated that *Staphylococcus aureus*, regardless of SE production, can damage the airway epithelial cells, thereby inducing the release of the alarmins IL-25, IL-33 and thymic stromal lymphopoietin (TSLP), which in turn can activate the type-2 innate lymphoid cells (ILC2) involved in IL-5-mediated eosinophilic inflammation.³⁷ The relationship between nasal SA colonization and asthma prevalence in adult patients has been demonstrated by a recent meta-analysis.³⁸

In conclusion, in the present study we have shown for the first time—at least to our knowledge—the ability of SEs to induce BAT-assessed basophil activation in vivo in more than one third of severe asthmatic patients. This finding further supports the pathogenetic role of SEs in severe asthma. Although further studies will be necessary to validate BAT as an assay to evaluate the sensitization state to SEs, we suggest to investigate the SE involvement by means of specific IgE detection and/or BAT in

	SEA*	SEB**	TSST***	P*	P**	P***
SE/Negative control (SI)						
FEV ₁						
Pre	-0.328	-0.382	-0.310	.013	.003	.019
Post	-0.289	-0.465	-0.298	.049	.001	.042
FVC						
Pre	-0.327	-0.415	-0.357	.013	.001	.006
Post	-0.344	-0.420	-0.285	.018	.003	.052
ACT	-0.297	-0.332	-	.025	.012	-
Asthma exacerbations	0.362	-	0.299	.011	-	.039
SE/anti-IgE						
FEV ₁ /FVC	0.008	0.140	0.054	.956	.355	.723

Note: Correlations are expressed by the Pearson's Correlation coefficient.

Abbreviations: ACT, Asthma control test; SE, *Staphylococcus aureus* enterotoxin; SEA, *Staphylococcus aureus* enterotoxins A; SEB, *Staphylococcus aureus* enterotoxins B; SI, stimulation index; TSST, toxic shock syndrome toxin 1.

* correlation between SEA-SI with functional and clinical parameters; ** correlation between SEB-SI with functional and clinical parameters; *** correlation between TSST-SI with functional and clinical parameters.

TABLE 3 Correlations of BAT stimulation indexes

severe asthmatic patients, especially in those with negative skin prick tests and high levels of total serum IgE.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

The original conception and design of the study came from CC. CC and FM took the responsibility for the whole content of the study and contributed to the writing of the manuscript. PF and GF performed the basophil activation test and contributed to its interpretation. AS performed serum SEs IgE measurement. MR, MaMa and MM managed data collection and analysis. TE and MM provided expertise in rhinolaryngoscopy. MaMa took the responsibility of study statistic. All authors have read, written and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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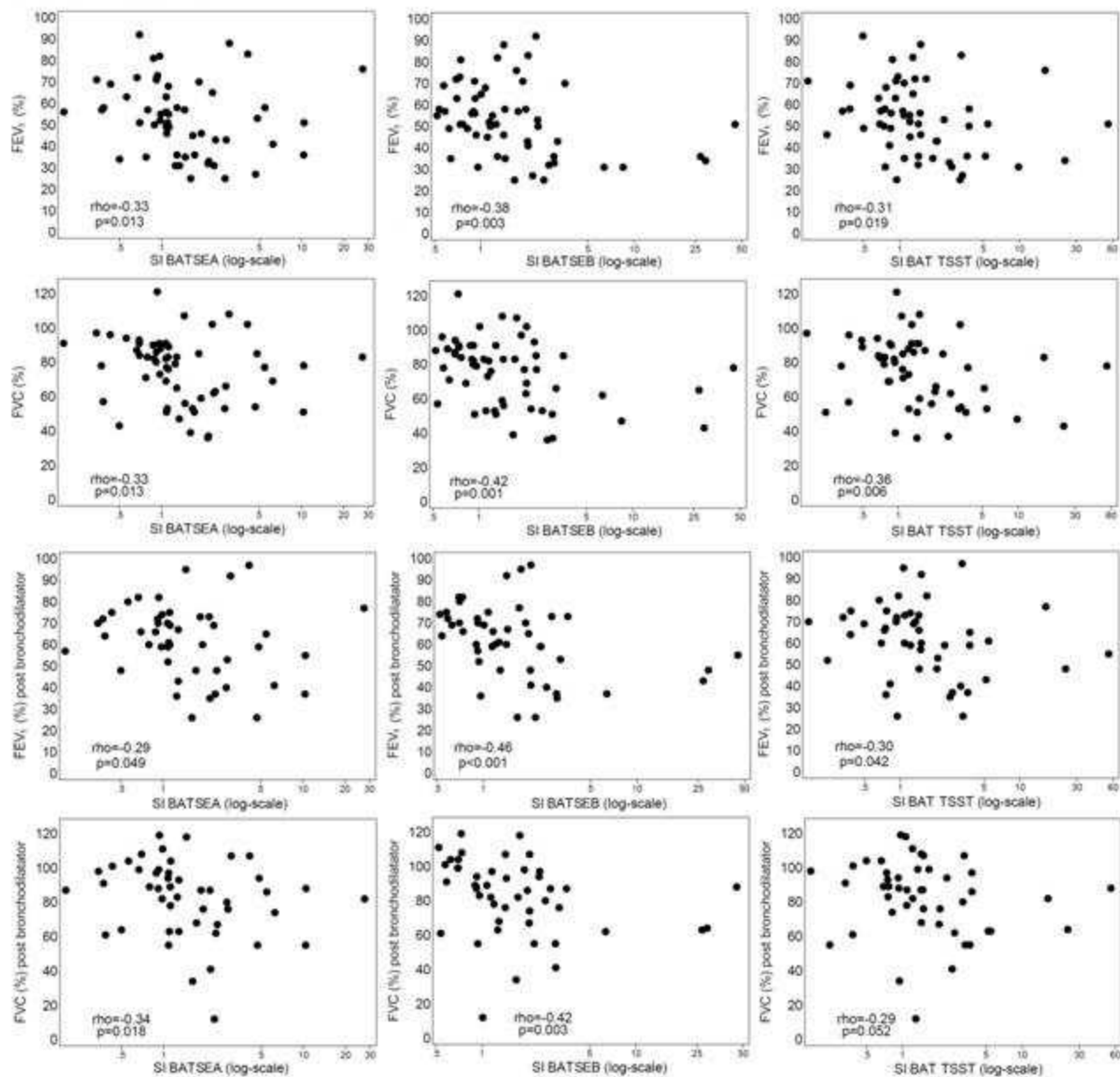
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SUPPORTING INFORMATION

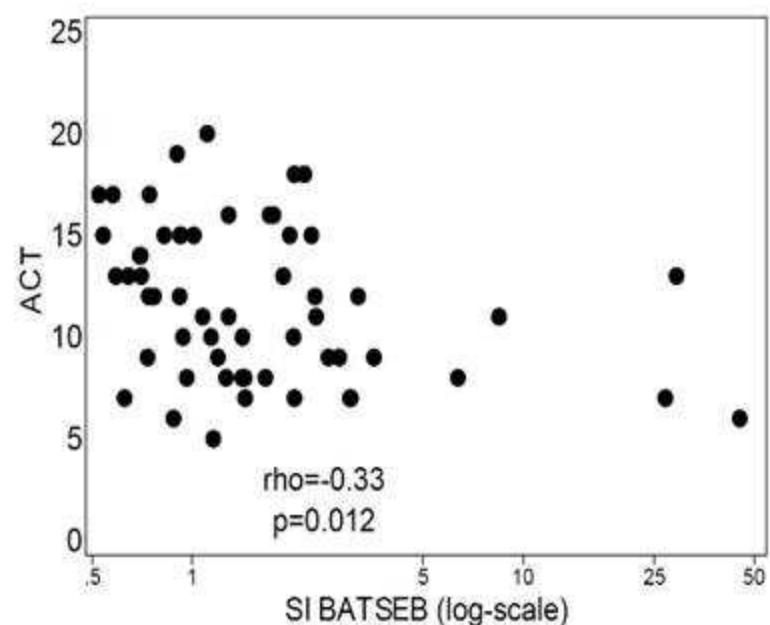
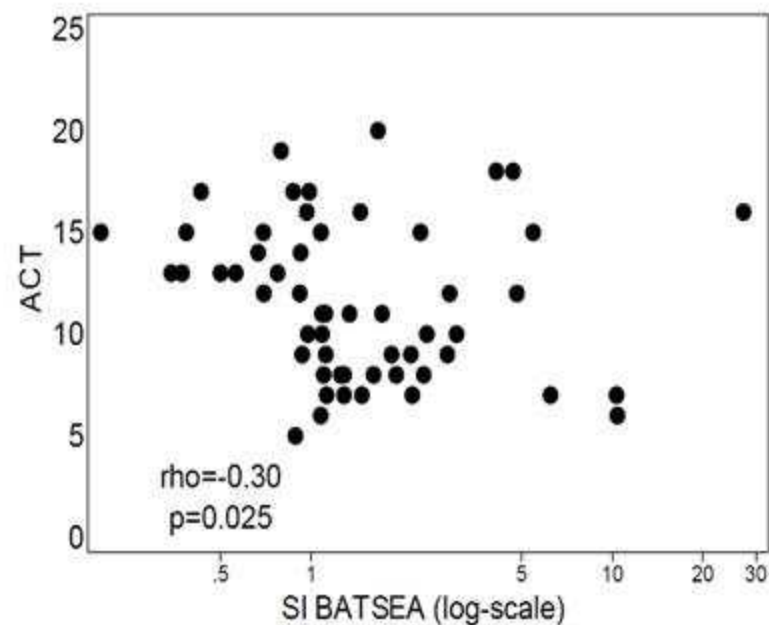
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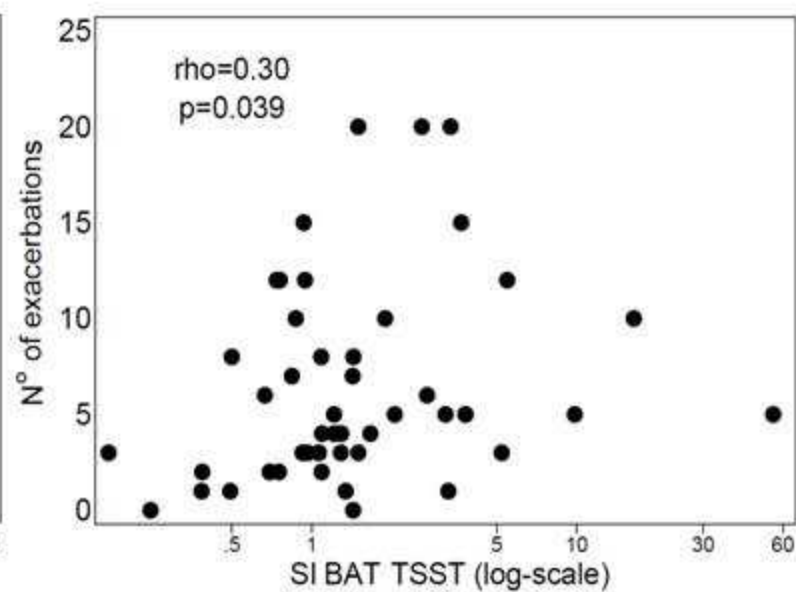
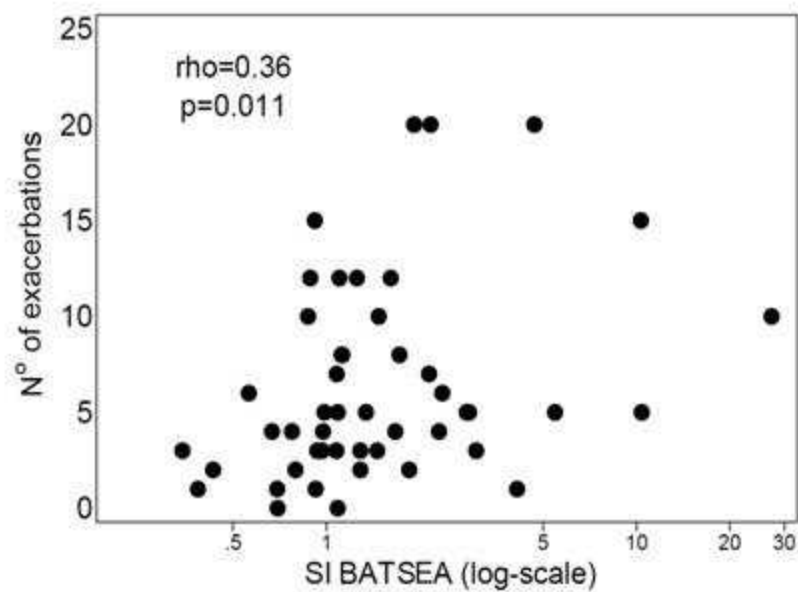
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4

