Activated non-neuronal cholinergic system correlates with non-type 2 inflammation and exacerbations in severe asthma

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Abstract

Background

Non-neuronal cholinergic system (NNCS) contributes to various inflammatory airway diseases. However, the role of NNCS in severe asthma (SA) remains largely unexplored.

Objective

To explore airway NNCS in SA.

Methods

In this prospective cohort study based on the Australasian Severe Asthma Network in a real-world setting, patients with SA (n = 52) and non-SA (n = 104) underwent clinical assessment and sputum induction. The messenger RNA (mRNA) levels of NNCS components and proinflammatory cytokines in the sputum were detected using real-time quantitative polymerase chain reaction, and the concentrations of acetylcholine (Ach)-related metabolites were evaluated using liquid chromatography coupled with tandem mass spectrometry. Asthma exacerbations were prospectively investigated during the next 12 months. The association between NNCS and future asthma exacerbations was also analyzed.

Results

Patients with SA were less controlled and had worse airway obstruction, a lower bronchodilator response, higher doses of inhaled corticosteroids, and more add-on treatments. The sputum mRNA levels of NNCS components, such as muscarinic receptors M1R - M5R, OCT3, VACHT , and ACHE ; proinflammatory cytokines; and Ach concentration in the SA group were significantly higher than those in the non-SA group. Furthermore, most NNCS components positively correlated with non-type (T) 2 inflammatory profiles, such as sputum neutrophils, IL8 , and IL1B . In addition, the mRNA levels of sputum M2R, M3R, M4R, M5R , and VACHT were independently associated with an increased risk of moderate-to-severe asthma exacerbations.

Conclusion

This study indicated that the NNCS was significantly activated in SA, leading to elevated Ach and was associated with clinical features, non-T2 inflammation, and future exacerbations of asthma, highlighting the potential role of the NNCS in the pathogenesis of SA.

Clinical Trial Registration

ChiCTR-OOC-16009529 (http://www.chictr.org.cn).

Introduction

Asthma is an important chronic respiratory disease with increasing prevalence in many countries. 1 Although inhaled corticosteroid (ICS) and long-acting β 2 -agonist (LABA) therapies can effectively treat most patients with asthma, a substantial proportion of them remain difficult to treat, especially those experiencing severe asthma (SA). 123 SA has proven to be a disease with high heterogeneity manifesting as complex and diverse pathophysiological features, clinical manifestations, and adverse asthma outcomes, 2 , 4 , 5 which poses a heavy ongoing burden for the health care resources. 5 , 6

Acetylcholine (Ach) is an endogenous neurotransmitter for both the peripheral and central nervous systems, which is released by the vagal nerve endings in the lung and increases the airway smooth muscle tone through activating muscarinic receptors (MRs), representing the potential roles of the cholinergic system in airway disease. 7 In addition, Ach can be synthesized and secreted by non-neuronal cells, termed as the non-neuronal cholinergic system (NNCS). 789 The NNCS is actually a system of all related protein molecules involved in the transportation, synthesis, release, reception, and breakdown of Ach throughout metabolism. 8 The NNCS includes MRs and Ach-related proteins that exist in the human airways and contribute to an increased cholinergic tone of the lung. 7 Activation of the NNCS in the airway can induce the downstream signaling of Ach, resulting in bronchoconstriction, airway inflammation, mucus hypersecretion, and airway remodeling, which can be observed in a number of airway diseases, including asthma. 1011121314

Several large clinical trials have revealed that inhaled bronchodilators with long-acting muscarinic antagonist (LAMA) treatment can prevent or reduce Ach-mediated contraction of the airway smooth muscle. 10 , 151617 Therefore, LAMAs are now used as an add-on treatment in SA on step 5 of Global Initiative for Asthma (GINA). 1 Moreover, increasing evidence from cell culture and animal studies suggests that LAMAs could also have anti-inflammatory effects. 7 , 10 , 14 A previous study by Milara et al 18 revealed that the expression of NNCS was up-regulated in the blood and sputum neutrophils in chronic obstructive pulmonary disease and aclidinium bromide could effectively reduce the release of cytokines and metalloproteinase in neutrophils in patients with chronic obstructive pulmonary disease. Nevertheless, no studies have explored whether the NNCS activated in SA and was involved in elevated inflammation and risk of asthma exacerbations.

Therefore, we aimed to investigate whether the NNCS (1) in the airway is activated in SA, (2) is associated with the clinical inflammatory characteristics of SA, and (3) correlates with future exacerbations of asthma.

Methods

Study Design and Participants

This was a prospective cohort study with a 12-month follow-up period based on the Severe Asthma Web-based Database from the Australasian Severe Asthma Network in a real-world setting. 19 The study was approved by the institutional review board at West China Hospital, Sichuan University (Chengdu, People's Republic of China) (No. 2014-30), and registered as ChiCTR-OOC-16009529 (http://www.chictr.org.cn).

Patients with asthma aged more than or equal to 18 years with stable asthma were consecutively recruited from the Asthma Clinic of West China Hospital at Sichuan University, People's Republic of China. Asthma was diagnosed by respiratory physicians according to the GINA guidelines. 1 Stable asthma was defined as a condition with no respiratory tract infection, exacerbation, or change in maintenance therapy in the past 4 weeks. 20 , 21 All patients provided written informed consent before participation. Detailed information on asthma diagnosis and the inclusion and exclusion criteria are provided in the Methods section of this article's eAppendix.

Definition of Severe Asthma

For treatment-based SA classification, GINA guidelines 1 were used to confirm the definition of SA. SA was defined as asthma that requires treatment with steps 4/5 (medium or high dose of ICS/LABA ± add-on) to prevent it from becoming “uncontrolled” or which remains “uncontrolled” despite this treatment. 1 , 19 Detailed definition of SA is provided in the eAppendix.

Data Collection and Clinical Assessments

Baseline clinical data from eligible patients, including sociodemographic and clinical characteristics, such as age, sex, family history of asthma, medications, asthma symptoms, and history of exacerbations, were collected. Asthma control and quality of life were assessed using the GINA Levels of Asthma Control 1 and Asthma Quality of Life Questionnaire, 21 respectively. On the basis of the American Thoracic Society/European Respiratory Society standards, fractional exhaled nitric oxide and spirometry were performed using the NIOX analyzer (Aerocrine, Solna, Sweden) and spirometer (model 6200; SensorMedics Corporation, Yorba Linda, California). 212223 Furthermore, all participants underwent sputum induction in the morning of study entry.

Sputum Induction, Processing, and Analysis

Sputum induction, processing, and analysis were performed as previously described. 242526 Total and differential sputum cell counts and cytokine levels were measured. Detailed information is provided in the eAppendix.

Sputum cellular phenotypes were classified as follows: eosinophilic asthma (EA, eosinophils ≥ 3% and neutrophils < 61%), neutrophilic asthma (neutrophils ≥ 61% and eosinophils < 3%), mixed granulocytic asthma (eosinophils ≥ 3% and neutrophils ≥ 61%), and paucigranulocytic asthma (eosinophils < 3% and neutrophils < 61%), based on the presence of sputum granulocytes. 25262728

RNA Extraction, Complementary DNA Synthesis, and Real-Time Quantitative Polymerase Chain Reaction

Levels of NNCS components (MRs [M1R to M5R] and proteins related to Ach synthesis [CHAT and CHT1], secretion [VACHT and OCT1 to OCT3], and hydrolysis [ACHE and BCHE]) and type (T) 2 or non-T2 proinflammation cytokines (interleukin [IL]-5, IL-6, IL-8, IL-1β, and tumor necrosis factor α) in induced sputum were detected using real-time quantitative polymerase chain reaction (RT-qPCR). Detailed information on the genes measured in this study is provided in eTable 1 of the eAppendix. Total RNA was isolated from the induced sputum and reverse transcribed to complementary DNA. RT-qPCR was performed using TaqMan reagents (Applied Biosystems, Foster City, California) as previously described. 26 , 29 Details are provided in the eAppendix.

Acetylcholine and Its Related Metabolites Detected by Liquid Chromatography Coupled With Tandem Mass Spectrometry

According to the procedure described in our previous studies, 30 , 31 Ach and its related metabolites in induced sputum samples were assayed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) using an EXION LC System (SCIEX) coupled with SCIEX 6500 QTRAP + Triple Quadrupole mass spectrometry (SCIEX) in multiple reaction monitoring mode. The methodologic details for sample preparation and LC-MS/MS are provided in the eAppendix.

Follow-Up and Management Strategies

To assess the occurrence of asthma exacerbations, patients received standardized treatment and were observed (through face-to-face visits or telephone calls, if unavailable) for 12 months. As a real-world clinical study, indications for patient treatment were based on the GINA guidelines from study entry to the end of the 12-month follow-up period. 1 Step-up or step-down treatments were adjusted in a continuous cycle of assessment, treatment, and review as recommended by the GINA guidelines. 1 As described in our previous study, to ensure reliability, the patients in this study were diagnosed, treated, and managed by qualified consulting physicians who had received standardized clinical training at our hospital. 20

Asthma Exacerbations

Asthma outcomes included moderate-to-severe exacerbations leading to unplanned and emergency department (ED) visits and hospitalizations. 24 Asthma exacerbations were reviewed according to the American Thoracic Society/European Respiratory Society statement. 32 For patients with multiple exacerbations during the 12-month follow-up, 2 courses separated by at least 1 week were recorded as separate asthma exacerbations. Detailed definitions of asthma exacerbations are provided in the eAppendix.

Statistical Analysis

Clinical data were analyzed using IBM SPSS software (version 21.0; SPSS, Chicago, Illinois) and reported as mean ± SD for normally distributed data and median (IQR) for nonparametric data. Categorical data were presented as counts and percentages (%). If possible, all data were normally distributed. Continuous variables were analyzed using Student's t test or Mann–Whitney U test for data with normal or non-normal distribution, respectively. Categorical data were compared using the χ 2 test or Fisher's exact test. Correlations were assessed using the Spearman's coefficient test. Logistic regression and negative binomial regression models were used to explore the relationship between the NNCS components and asthma future exacerbations, adjusting for sex, age, body mass index (BMI), ICS/LABA, LAMA, and baseline forced expiratory volume in 1 second (FEV 1)% predicted. The adjusted odds ratio (ORadj) with a 95% CI was calculated. All tests are 2-tailed, and statistical significance was set at P less than or equal to .05.

Results

Demographic and Characteristics of the Patients Enrolled in the Cohort

The included patients were divided into the SA (n = 52) and non-SA (n = 104) groups. Compared with the non-SA group, the SA group had a longer asthma duration (10.00 [5.00, 26.00] vs 4.00 [1.00, 13.75] years, P < .001), worse asthma control according to the GINA classification (P = .001), lower quality of life (Asthma Quality of Life Questionnaire, 5.74 ± 0.87 vs 6.25 ± 0.64, P < .001), and greater asthma symptoms (visual analog scale score of dyspnea, cough, wheezing, and chest tightness, all P < .05). Regarding asthma medication use, a greater proportion of patients with SA received ICS plus LABA (100% vs 73.1%, P < .001), theophylline (26.9% vs 10.6%, P = .009), or a leukotriene receptor antagonist (88.5% vs 54.8%, P < .001). In addition, patients with SA received more intensive ICS therapy (beclomethasone dipropionate equivalent: 1000 [1000, 2000] vs 400 [400, 400] μg/d, P < .001). None of the patients in either group underwent biologic therapy. Furthermore, the patients in the SA group had worse lung function (presented as pre-FEV 1 L, pre-FEV 1 % predicted, and pre-FEV 1 /forced vital capacity %, all P < .05) and poor bronchodilatation (ΔFEV 1 mL) (Table 1). No statistically significant differences were observed in age, sex, BMI, smoking, family history of asthma, age of asthma onset, LAMA use, oral corticosteroid use, fractional exhaled nitric oxide, or atopy between the 2 groups (all P > .05) (Table 1).

Table 1

Demographic and Clinical Characteristics of Patients With Asthma in SA and Non-SA Groups

Variables	SA	Non-SA	t/χ 2 / Z	P value
n	52	104		
Male, n (%)	21 (40.4)	40 (38.5)	0.054	.817
Age, y	50.17 ± 12.37	47.03 ± 12.61	1.477	.142
BMI, kg/m 2	23.02 ± 2.82	23.17 ± 3.27	−0.290	.772
Smoking, current/ex/non, n	3/11/38	9/20/75	0.444	.801
Family history of asthma, n (%)	14 (27.5)	27 (26.0)	0.039	.843
Age of asthma onset, y	30.50 (23.00-46.75)	38.00 (25.25-49.00)	−0.820	.412
Asthma duration, y	10.00 (5.00-26.00)	4.00 (1.00-13.75)	−3.681	<.001
Asthma medications				
ICS/LABA, n (%)	52 (100.0)	76 (73.1)	17.063	<.001
ICS (BDP, μg/d)	1000 (1000-2000)	400 (400-400)	−9.102	<.001
LAMA, n (%)	5 (9.6)	5 (4.8)	0.654	.419
Theophylline, n (%)	14 (26.9)	11 (10.6)	6.883	.009
LTRA, n (%)	46 (88.5)	57 (54.8)	17.503	<.001
OCS, n (%)	1 (1.9)	0 (0)	-	.333 b
GINA controlled level, un/partially/controlled, n	22/24/6	16/60/28	14.938	.001
AQLQ, score	5.74 ± 0.87	6.25 ± 0.64	−3.751	<.001
Asthma symptoms				
Dyspnea, VAS	20 (0-40)	0 (0-20)	−2.455	.014
Cough, VAS	20 (0-40)	10 (0-30)	−1.978	.048
Wheezing, VAS	10 (0-30)	0 (0-10)	−3.023	.003
Chest tightness, VAS	0 (0-30)	0 (0-10)	−2.773	.006
Spirometry				
Pre-FEV 1 , L	1.89 ± 0.73	2.23 ± 0.78	−2.584	.011
Pre-FEV 1 , % predicted	68.11 ± 21.20	76.74 ± 19.81	−2.490	.014
Pre-FEV 1 /FVC, %	62.56 ± 13.49	67.80 ± 11.99	−2.452	.015
ΔFEV 1 , mL	140 (90-270)	280 (120-380)	−2.530	.011
ΔFEV 1 , %	9.85 (4.62-17.26)	13.32 (5.77-18.98)	−1.295	.195
FeNO, ppb	31.50 (11.00-64.00)	26.00 (16.25-49.50)	−0.576	.564
Atopy a , n (%)	23 (46.0)	58 (56.3)	1.436	.231

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Abbreviations: AQLQ, asthma quality of life questionnaire; BDP, beclomethasone dipropionate; BMI, body mass index; FeNO, fractional exhaled nitric oxide; FEV 1 , forced expiratory volume in 1 second; FVC, forced vital capacity; GINA, Global Initiative for Asthma; ICS, inhaled corticosteroid; LABA, long-acting β 2 -agonist; LAMA, long-acting muscarinic antagonist; LTRA, leukotriene receptor antagonist; OCS, oral corticosteroid; SA, severe asthma; VAS, visual analog scale.

P values < 0.05 were bolded.

a Atopy was defined by a positive skin prick testing result.

b P value of the Fisher exact test.

Sputum Inflammation Characteristics

Compared with non-SA patients, non-T2 cytokines such as IL6 (2.15 [0.32, 5.86] vs 0.82 [0.33, 1.91], P = .048), IL8 (1.11 [0.49, 3.00] vs 0.80 [0.35, 1.98], P = .018), and IL1B (0.99 [0.49, 4.62] vs 0.76 [0.40, 1.71], P = .030) in induced sputum were expressed at higher levels in the SA group. No differences were observed in total and inflammatory cell counts in the induced sputum (Table 2).

Table 2
Sputum Inflammation Characteristics of Patients With Asthma in SA and Non-SA Groups

Variables	SA	Non-SA	t/χ 2 / Z	P value
n	52	104		
Induced sputum cells				
Total cell count, × 10 6 /mL	0.28 (0.16-0.51)	0.31 (0.17-0.53)	−0.262.794	
Neutrophils				
Count × 10 4 /mL	8.58 (4.50-27.79)	11.84 (2.24-30.25)	−0.186.852	
%	39.63 (18.38-67.52)	43.88 (15.00-73.94)	−0.294.769	
Eosinophils				
Count × 10 4 /mL	0.01 (0.00-0.68)	0.03 (0.00-0.46)	−0.142.887	
%	0.13 (0.00-1.75)	0.25 (0.00-1.25)	−0.056.955	
Inflammatory phenotypes				
EA/NA/MGA/PGA, n	6/12/2/18	11/34/6/43	0.586	.900
Expression of cytokines measured by RT-qPCR in induced sputum				
IL5	1.27 (0.24-10.25)	0.91 (0.17-3.92)	1.273	.205
IL6	2.15 (0.32-5.86)	0.82 (0.33-1.91)	1.992	.048
IL8	1.11 (0.49-3.00)	0.80 (0.35-1.98)	2.396	.018
IL1B	0.99 (0.49-4.62)	0.76 (0.40-1.71)	2.191	.030
TNF	1.17 (0.52-3.19)	0.74 (0.51-1.66)	1.555	.122

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Abbreviations: EA, eosinophilic asthma; IL, interleukin; MGA, mixed granulocytic asthma; NA, neutrophilic asthma; PGA, paucigranulocytic asthma; RT-qPCR, real-time quantitative polymerase chain reaction; TNF, tumor necrosis factor; SA, severe asthma. P values < 0.05 were bolded.

Activated Non-Neuronal Cholinergic System Components in Induced Sputum From Patients With Severe Asthma

The messenger RNA (mRNA) of MR subtypes and Ach-related proteins were expressed in induced sputum cells (Fig. 1). Compared with patients in the non-SA group, the MR subtypes M1R to M5R in sputum cells were expressed at higher levels in the SA group (all P < .05) (Fig. 1 A-E). OCT3, the transmembrane protein that transports Ach, was increased in patients with SA (P = .001) (Fig. 1 G), whereas OCT1 did not increase (Fig. 1 F) and OCT2 was undetectable. VACHT, which is responsible for loading Ach into secretory organelles, and ACHE, an Ach hydrolase, were expressed at higher levels in induced sputum from patients with SA (all P < .05) (Fig. 1 I and K), whereas BCHE was undetectable. CHT1, a high-affinity choline transporter that transports choline, had an upward trend in patients with SA (P = .081) (Fig. 1 J) . CHAT, the enzyme responsible for

catalyzing Ach synthesis, had no difference between the SA and non-SA groups (Fig. 1 H).

mRNA levels of NNCS components in induced sputum from patients with SA (n = 52) and non-SA (n = 104). The horizontal line in the box indicates the median values of muscarinic receptors (A-E) and proteins related to Ach synthesis, secretion, and hydrolysis (F-K); box indicates the IQR; and vertical lines indicate the 5 to 95 percentile. ACHE, acetylcholinesterase; CHAT, choline acetyltransferase; CHT, high-affinity choline transporter; MR, muscarinic receptor; mRNA, messenger RNA; NNCS, non-neuronal cholinergic system; OCT, organic cation transporter; SA, severe asthma; VACHT, vesicular acetylcholine transporter.
We measured the levels of Ach and its related metabolites in induced sputum samples using LC-MS/MS. The demographic and clinical characteristics of patients with asthma are presented in eTable 2 . Compared with non-SA patients (n = 80), patients with SA (n = 40) had significantly elevated Ach levels (P = .032) (Fig. 2 A), whereas no difference was observed in phosphorylcholine, glycerophosphocholine, and pyruvate levels (P > .05) (Fig 2 B-D).

Concentrations of Ach-related metabolites in induced sputum from patients with SA (n = 40) and non-SA (n = 80). The horizontal line in the box indicates the median value; box indicates the IQR; and vertical lines indicate the 5 to 95 percentile. Ach, acetylcholine; Chop, phosphorylcholine; GPC, glycerophosphocholine; SA, severe asthma.
Correlations Between Non-Neuronal Cholinergic System Components and Clinical Characteristics of Patients With Asthma

Correlation analyses revealed that M2R and ACHE were positively correlated with current age and age of asthma onset (all P < .05) (Fig 3 and eTable 3). The cholinergic MR subtypes (M1R - M5R), Ach-related secretion, and hydrolysis proteins (OCT3, VACHT , and ACHE) were positively correlated with ICS dosage (all P < .05), whereas M1R (r = −0.206), M3R (r = −0.158), OCT3 (r = −0.232), CHAT (r = −0.266), VACHT (r = −0.217), CHT1 (r = −0.226), and ACHE (r = −0.202) were negatively correlated with GINA control levels (all P < .05). Furthermore, M1R, M2R, M5R, OCT1, VACHT , and ACHE positively correlated with cough intensity (all P < .05) (Fig 3 and eTable 3).

Heatmap of correlations between NNCS components and clinical characteristics of asthma. ACHE, acetylcholinesterase; AQLQ, asthma quality of life questionnaire; BDP, beclomethasone dipropionate; BMI, body mass index; CHAT, choline acetyltransferase; CHT, high-affinity choline transporter; FeNO: fractional exhaled nitric oxide; FEV 1 , forced expiratory volume in 1 second; FVC, forced vital capacity; GINA, Global Initiative for Asthma; ICS, inhaled corticosteroids; IL, interleukin; MR, muscarinic receptor; NNCS, non-neuronal cholinergic system; OCT, organic cation transporter; TNF, tumor necrosis factor; VACHT, vesicular acetylcholine transporter; VAS, visual analog scale.

In addition, NNCS components, such as cholinergic MR subtypes (M1R - M5R), Ach-related secretion, and hydrolysis proteins (OCT1, OCT3, VACHT , and ACHE) were negatively correlated with bronchodilator response (ΔFEV 1) (all P < .05) (Fig 3 and eTable 3).

Correlations Between Non-Neuronal Cholinergic System Components and Sputum Inflammation

In induced sputum, the mRNA levels of M1R, M2R, M5R, OCT1 , and ACHE were positively correlated with the sputum neutrophil percentage (r = 0.178, r = 0.239, r = 0.196, r = 0.260, and r = 0.186, respectively) (all P < .05), whereas OCT3 was negatively correlated with the sputum neutrophil percentage (r = −0.292). In terms of the mRNA expression levels of airway cytokines, most NNCS components were positively correlated with non-T2 inflammation factors, which mainly presented as IL8 was positively correlated with M1R (r = 0.351), M2R (r = 0.267), M4R (r = 0.304), M5R (r = 0.251), OCT1 (r = 0.439), CHAT (r = 0.182), VACHT (r = 0.386), CHT1 (r = 0.200), and ACHE (r = 0.305), whereas IL1B was positively correlated with M1R (r = 0.286), M2R (r = 0.224), M4R (r = 0.248), M5R (r = 0.241), OCT1 (r = 0.373), VACHT (r = 0.315), and ACHE (r = 0.258) (all P < .05). In addition, IL5 positively correlated with M1R (r = 0.160) and OCT3 (r = 0.252) (all P < .05) (Fig 3 and eTable 4).

Asthma Exacerbations Within the 12-Month Follow-Up

A total of 154 patients (98.7%) completed the 12-month follow-up. Compared with the non-SA cohort, the SA cohort experienced more moderate-to-severe exacerbations (51.0% vs 12.6%, P < .001). In addition, the frequency of moderate-to-severe exacerbations was higher in the SA cohort than in the non-SA cohort (1.02 ± 1.41 vs 0.19 ± 0.58, P < .001). Furthermore, patients with SA had a higher proportion and frequency of hospitalizations (11.8% vs 2.9%, P = .028; 0.22 ± 0.64 vs 0.03 ± 0.17, P = .024) and unplanned visits (31.4% vs 7.8%, P < .001; 0.59 ± 1.19 vs 0.15 ± 0.55, P < .001) respectively, than non-SA ones (Table 3).

Table 3
Asthma Exacerbations in the Next 12 Months Among Patients With Asthma in SA and Non-SA Groups

Variables	SA	Non-SA	t/χ 2 / Z	P value
n	51	103		
Moderate-to-severe exacerbation				
n (%)	26 (51.0)	13 (12.6)	26.540	<.001
Mean ± SD	1.02 ± 1.41	0.19 ± 0.58	−5.219	<.001
Hospitalization				
n (%)	6 (11.8)	3 (2.9)	4.857	.028
Mean ± SD	0.22 ± 0.64	0.03 ± 0.17	−2.252	.024
ED visit				
n (%)	4 (7.8)	2 (1.9)	-	.094 a
Mean ± SD	0.08 ± 0.27	0.02 ± 0.14	−1.775	.076
Unplanned visit				

n (%) 16 (31.4) 8 (7.8) 14.448<.001
Mean ± SD 0.59 ± 1.19 0.15 ± 0.55 −3.713<.001
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Abbreviations: ED, emergency department; SA, severe asthma.
P values < 0.05 were bolded.
a P value of the Fisher exact test.

Associations Between Non-Neuronal Cholinergic System Components and Asthma Exacerbations

After adjusting for relevant confounders such as sex, age, BMI, ICS/LABA, LAMA, and baseline FEV 1 % predicted, the logistic regression analysis revealed that M2R (ORadj = 2.198, 95% CI = [1.250, 3.865]), M3R (ORadj = 1.762, 95% CI = [1.030, 3.094]), M4R (ORadj = 1.846, 95% CI = [1.054, 3.232]), M5R (ORadj = 2.298, 95% CI = [1.297, 4.073]), and VACHT (ORadj = 2.161, 95% CI = [1.212, 3.854]) were associated with an increased risk of moderate-to-severe asthma exacerbations at the 12 months of follow-up (all P < .05). M1R, M2R, M5R , and VACHT were correlated with a higher risk of ED visits, whereas M4R, OCT1 , and VACHT were correlated with an increased risk of unplanned outpatient visits (all P < .05). No relationship was found between NNCS components and hospitalizations (all P > .05) (Fig 4 A and eTable 5).

Relationship between NNCS components and asthma exacerbations within the 12-month follow-up period using (A) logistic regression and (B) negative binomial regression models. Adjusted for sex, age, BMI, ICS/LABA, LAMA, and baseline FEV 1 % predicted. ACHE, acetylcholinesterase; BMI, body mass index; CHAT, choline acetyltransferase; CHT, high-affinity choline transporter; FEV 1 , forced expiratory volume in 1 second; ICS, inhaled corticosteroid; LABA, long-acting β 2 -agonist; LAMA, long-acting muscarinic antagonist; MR, muscarinic receptor; NNCS, non-neuronal cholinergic system; OCT, organic cation transporter; OR, odds ratio; VACHT, vesicular acetylcholine transporter. P values < 0.05 were bolded.
Furthermore, in terms of asthma exacerbation frequency, the negative binomial regression analysis revealed that M1R, M2R, M3R, OCT1 , and CHT1 correlated with higher ED visit frequency (ORadj = 5.520, 95% CI = [1.148, 26.546]), increased hospitalization (ORadj = 2.730, 95% CI = [1.144, 6.513]) and ED visit (ORadj = 5.316, 95% CI = [1.135, 24.885]) frequency, increased moderate-to-severe exacerbation (ORadj = 1.586, 95% CI = [1.045, 2.409]) and hospitalization (ORadj = 2.946, 95% CI = [1.163, 7.460]) frequency, increased frequencies of moderate-to-severe exacerbations (ORadj = 1.558, 95% CI = [1.012, 2.400]) and unplanned outpatient visits (ORadj = 1.818, 95% CI = [1.003, 3.294]), and higher hospitalization frequency (ORadj = 3.077, 95% CI = [1.078, 8.786]) (all P < .05), respectively (Fig 4 B and eTable 6).

Discussion

To the best of our knowledge, this is a pilot prospective cohort study conducted in a real-world setting to explore the relationships between NNCS and disease severity,

clinical characteristics, airway inflammation, and future exacerbations of SA. The NNCS components, including MRs, OCT3, VACHT , and ACHE at the mRNA level and Ach at the metabolic level, were significantly increased in induced sputum from patients with SA. Moreover, the up-regulated NNCS components observed in SA were related to the clinical characteristics of asthma, such as age, worse asthma control and lung function, more intense coughing, and non-T2 inflammation. In the next 12 months, some NNCS components (M2R, M3R, M4R, M5R , and VACHT) were associated with a higher risk of future asthma exacerbations.

Similar to the findings of previous studies, 5 , 19 , 33 , 34 patients with SA had a longer asthma duration, worse asthma control, lower quality of life, and reduced lung function and bronchodilator response than those with non-SA. We also found that patients with SA experienced more non-T2 inflammation, which was manifested by the higher expression of non-T2 inflammation markers such as IL8 and IL1B in this study. Moreover, patients with SA had a significantly increased risk of exacerbations during the 12-month follow-up period. These clinical characteristics are related to the physiological and pathologic features of SA, which may be explained by genetic factors, high heterogeneity of airway inflammation, severe airway remodeling, and corticosteroid insensitivity. 2

The NNCS of the airways has been discussed as an interesting drug target for anticholinergic treatment. 7 The NNCS in the sputum of patients with asthma has been implicated in the pathogenesis of the disease. 7 , 14 , 35 The NNCS contains multiple components, including MRs and various Ach-related proteins, which have different functions in the transportation, synthesis, release, reception, and breakdown of Ach (eTable 1). In this study, we revealed that MR subtypes and Ach-related proteins, particularly M1R to M5R, OCT3, VACHT, ACHE , and Ach, were elevated in induced sputum from patients with SA. These findings indicate that non-neuronal cells in the airways of patients with asthma can also synthesize Ach, which is secreted through VACHT and OCT3 and acts on MRs. Ach may participate in the pathophysiology and pathogenesis of SA, including the airway wall contractility, hyperresponsiveness, inflammation, and structural changes in the airways. 10 , 12 , 36

Previously published studies have revealed that Ach signaling increased in the pathophysiology of asthma. 101112 Ach binds to airway MRs to trigger smooth muscle contraction and mucus secretion, mediates inflammation by inducing chemotaxis and the release of inflammatory mediators of inflammatory cells, and participates in airway remodeling. 101112 , 37 These pathologic changes ultimately affect the clinical manifestations and outcomes in patients with asthma. Notably, in our study, we found that patients with SA had a lower bronchodilator response, which was negatively correlated with NNCS, indicating an association between more severe airway remodeling and NNCS in SA. Furthermore, MR expression negatively correlated with the level of asthma control and positively correlated with cough intensity, sputum neutrophil percentage, and non-T2 cytokines (IL8 and IL1B), potentially indicating heightened activation of Ach signaling. Moreover, patients with SA experienced more non-T2 inflammation and future exacerbations in this study, and MRs were significantly correlated with IL8, IL1B , and asthma exacerbations. Our additional analysis between

the patients with EA and non-EA patients indicated that sputum OCT1 levels were higher in the non-EA group, which was classified as a non-T2 phenotype (data not revealed). Therefore, we speculated that MRs mediate future exacerbations of SA through the action of non-T2 cytokines such as IL8 and IL1B .

Large clinical trials have revealed the therapeutic benefits of LAMAs in airway smooth muscle relaxation through the inhibition of MR activity and are recommended by current guidelines for use as an important add-on treatment for SA. 1 , 36 , 3839404142 Our additional analysis revealed that NNCS expression did not differ between patients who used and those who did not use LAMA (data not revealed). Even though a small sample size of 10 patients was applied in the LAMA group, it still gave us the possible information that LAMAs target the MRs in the airway but did not affect the expression of NNCS, resulting in the inhibition of bronchoconstriction, airway remodeling, and potential inflammation inhibition. 10 , 43 , 44 This study indicated that the NNCS, including MRs, was highly expressed in SA and was correlated with airway inflammation and further exacerbations in asthma, suggesting a potential scientific rationale that the NNCS may be one of the novel potential treatable traits in the SA population. Further investigations are needed to clarify the mechanism of action of NNCS in the pathology of SA and to confirm the effects of LAMA on the levels of NNCS components.

This study had several limitations. First, this study lacked healthy participants as a control group; thus, we could not evaluate whether the NNCS in patients with non-SA would be considered high compared with healthy controls at baseline. We measured NNCS in an adult population with stable asthma. Therefore, further investigations are required to understand how expression levels change during exacerbations. Second, the protein levels of the NNCS components were not determined because of cell paucity in the induced sputum. However, the molecular assessment of NNCS component gene expression using RT-qPCR has some strengths. RT-qPCR is highly sensitive and allows for the quantification of rare transcripts. Moreover, it has high specificity, good reproducibility, and a wide dynamic range. 45 , 46 Third, the NNCS component levels were measured only once during the 1-year cohort study, which led to the inability to explore whether the NNCS level was an invariant characteristic despite asthma medication regimens. Fourth, whether Ach was completely derived from the NNCS could not be determined. However, we found that the Ach-related proteins in NNCS were highly expressed at the mRNA level in induced sputum from patients with SA, indicating that the increase in Ach was partially caused by NNCS. Finally, although we found that NNCS components were up-regulated in patients with SA at the mRNA level, further investigation is needed to determine whether LAMAs can reduce the levels of NNCS components.

In conclusion, we found that the NNCS was activated in patients with SA and was associated with worse asthma control and bronchodilator response. Meanwhile, NNCS positively correlated with non-T2 inflammatory signatures and a higher risk of moderate-to-severe asthma exacerbations, which provides insight into the rationale for treating patients with SA with LAMAs through anti-inflammatory effects. This study suggested that NNCS, as a novel potentially treatable trait of SA, could provide more

potential clinical implications for asthma treatment. Further studies are needed to assess whether monitoring the levels of NNCS components can facilitate more accurate and successful clinical interventions.