

ORIGINAL ARTICLE

Rhinitis, Sinusitis, and Upper Airway Disease

Artemisia annua-sublingual immunotherapy for seasonal allergic rhinitis: A randomized controlled trial

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Abstract

Background: *Artemisia annua* is an important autumnal pollen allergen for seasonal allergic rhinitis (SAR) in northern China. To date, no study has investigated allergen immunotherapy with *A annua*. We aimed to investigate the efficacy and mechanisms underlying *A annua*-sublingual immunotherapy (SLIT).

Methods: This was a randomized, double-blind, placebo-controlled phase III clinical trial involving 71 SAR patients, randomized to SLIT with *A annua* extract (n = 47) or placebo (n = 24) for 32 weeks. Total nasal symptom score (TNSS; primary clinical end point) was evaluated at baseline (peak pollen phase (PPP) in the previous year), initiation of *A annua*-SLIT, 1st PPP during SLIT, end of SLIT and 2nd PPP during follow-up. Blood samples and nasal secretions were collected at beginning and after SLIT for assessment of T cells and inflammatory mediators. Safety was assessed according to adverse events (AEs) reported.

Results: *Artemisia annua*-SLIT significantly reduced TNSS to a greater level from baseline (from 9.45 ± 1.68 to 6.16 ± 2.27) than placebo (from 9.29 ± 2.09 to 9.05 ± 2.40) at the 1st PPP ($P < .001$) and sustained the improvement in symptoms throughout to the 2nd PPP. Preseasonal *A annua*-SLIT for 16 weeks significantly decreased Th2 cells, increased nTreg and Tr1 cells in blood; and increased cystatin 1 (CST1) in nasal secretion after 16 and 32 weeks compared with pretreatment. Overall, 17/47 patients experienced mild local AEs and 2 patients mild systemic AEs, after *A annua*-SLIT.

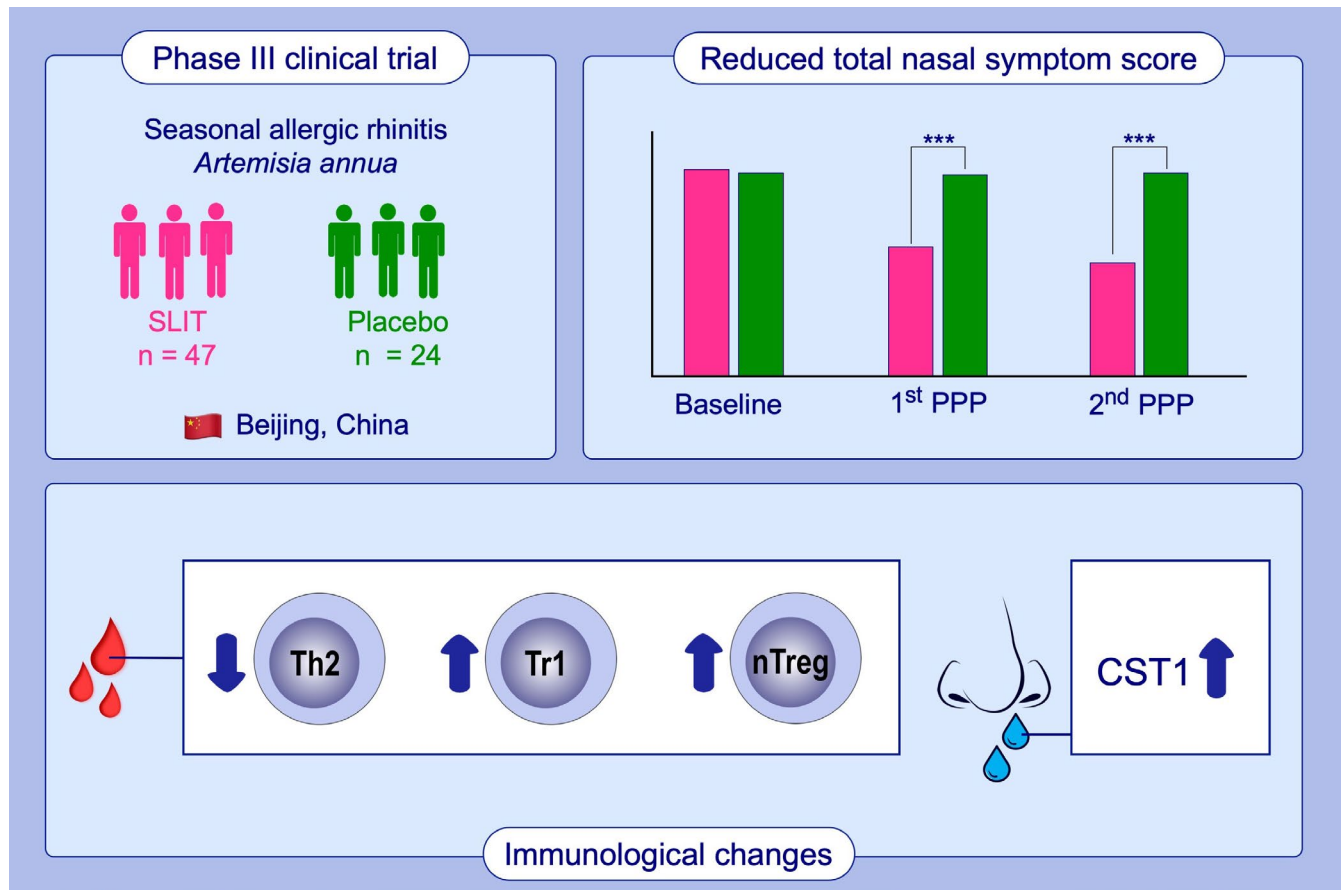
Conclusion: *Artemisia annua*-SLIT is an efficacious and safe treatment in patients with *A annua* SAR.

KEYWORDS

allergen immunotherapy (AIT), allergic rhinitis, *Artemisia annua*, efficacy, safety

Abbreviations: CST1, cystatin 1; PPP, peak pollen season; SLIT, sublingual immunotherapy.

Lou and Huang these authors contributed equally to this paper.



GRAPHICAL ABSTRACT

This randomized, double-blind, placebo-controlled phase III clinical trial demonstrates the efficacy and favourable safety profile of the 32-week SLIT with *Artemisia annua* in patients with seasonal allergic rhinitis. *Artemisia annua*-SLIT significantly reduces total nasal symptom score at the 1st and 2nd PPP compared with placebo. Preseasonal *Artemisia annua*-SLIT significantly decreases Th2 cells, increases nTreg and Tr1 cells in blood and increases CST1 in nasal secretion.

1 | INTRODUCTION

Allergic rhinitis (AR) is a global health issue, which affects 10%-40% of the population worldwide^{1,2} and significantly impacts the quality of life, resulting in a huge economic and psychological burden. The prevalence of self-reported AR in China has significantly increased.³ Traditionally, AR can be classified into seasonal AR (SAR) and perennial AR (PAR); and classically, outdoor aeroallergens are major causes for SAR.

Artemisia is mainly distributed in temperate, cold and subtropical regions of Asia, Europe and North America. *Artemisia* was generally considered as one of the most harmful sensitizing species for pollinosis during late summer and autumn in China, because of its production of pollen grain in large quantities.⁴ The available common species of *Artemisia* in different continents responsible for allergies are markedly distinctive; for example, *A vulgaris* in Europe and North America, *A annua*, *A sieversiana* and *A argyi* in China. The genus *Artemisia* includes 187 species in China,⁵ of which *A annua* grows widely in the northern China and is a major outdoor allergen responsible for allergic rhinitis (AR) and airway hyperresponsiveness.

Although several forms of treatments are available for AR, to date, allergen immunotherapy (AIT) is the only aetiological treatment for AR. Since the introduction of AIT in clinical practice^{6,7} nearly 110 years ago, the short-term and long-term efficacy of AIT has been well established through rigorous clinical trials.⁸⁻¹¹ Considering the potential risks of AIT-associated adverse events (AEs), sublingual immunotherapy (SLIT) has been the preferred route of allergen administration compared with subcutaneous immunotherapy (SCIT) because of its better safety profile and the convenience of self-administration without medical supervision.¹² However, presently it is not clear whether SLIT or SCIT is superior in efficacy because direct comparisons are lacking and existing data are controversial.^{13,14} Nevertheless, real-world studies have shown that SLIT can slow down AR/asthma progression and decrease asthma onset.¹⁵

A large number of clinical trials have confirmed that both SLIT and SCIT can significantly reduce AR symptoms and medication usage.⁸⁻¹¹ Moreover, the benefit of AIT was maintained for several years after discontinuation, with considerable long-term efficacy.^{16,17} Studies in western countries have mostly concentrated on AIT with grass pollens,^{18,19} whilst Japanese studies have concentrated on

pollen from Japanese cedar.²⁰ Recently, the biomarkers of responsiveness to AIT have aroused great attention, since some patients could remain unresponsive after AIT. A latest European Academy of Allergy and Clinical Immunology (EAACI) position paper summarized the seven domains of possible biomarkers on the clinical efficacy of AIT, including IgE, IgG subclasses, serum inhibitory activity for IgE, basophil activation, relative cytokines and chemokines, cellular markers and in vivo markers.²¹ However, to date, no consensus has been reached on reliable markers in this regard.

Considering the widespread and socio-economic impact of *Artemisia* in China, relatively little research²²⁻²⁴ has focused on the efficacy and safety of AIT with *A annua* allergen to date. The purpose of this study was to evaluate the efficacy and immunological changes following SLIT with *A annua* allergen extracts in adults with *A annua*-associated AR. In this respect, the present study is the first clinical *A annua*-SLIT trial approved by the Chinese Food and Drug Administration.

2 | METHODS

2.1 | Ethics statement

The study protocol (Figure S1) was approved by the Medical Ethics Committee of Beijing TongRen Hospital and Beijing Institute of Otolaryngology prior the enrolment of participants; each of whom gave written informed consent prior to performing any procedures. This study was registered at clinicaltrial.gov (NCT 03649139).

2.2 | Study design

This was a randomized, double-blind, placebo-controlled, single-centre clinical trial undertaken in the Department of Otolaryngology Head and Neck Surgery and Department of Allergy, Beijing TongRen Hospital. Subjects aged 18-60 years were recruited in April 2017 from the outpatients that visited our department between July 2016 and October 2016, and whose symptoms and medication scores were recorded in the database of our centre.

Recruitment criteria included a clinical history of at least 2 years of seasonal allergic rhinitis with/without asthma related to *A annua* and no previous AIT. The diagnosis of AR was based on the ARIA criteria in 2016²; including patients aged between 18 and 60 years, two or more nasal symptom scores ≥ 2 points during July to October in 2016. Sensitization to *A annua* was confirmed by the presence of specific immunoglobulin E (sIgE; ≥ 3.5 kU/L), using UniCAP system (Phadia). Patients with serum-specific IgE against ragweed or *Humulus* over 3.5 kU/L, which was higher than *A annua*, were excluded. Other exclusion criteria were shown in repository.

At run-in phase, demographic and clinical data were collected; and eligible participants were randomized in a ratio of 2:1

to receive either glycerinated *A annua* allergen drops as SLIT, or matched sublingual placebo drops for 32 weeks (16-week pre-seasonal treatment, 8-week treatment during pollen season and 8-week post-seasonal treatment). The *A annua* drops were manufactured and provided by Zhejiang Wolwo Bio-pharmaceutical Co., Ltd., as five vials of specific *A annua* extracts with increasing concentrations ranging from 25 to 16 000 BU/mL, and the placebo drops were physically identical to active medication, but without allergenic components. Drops were administered sublingually for 1 minute before swallowing.

Randomization, double-blinding and SLIT protocol are presented in greater detail in the Online Repository. Clinical end points (described below) were collected at the baseline (peak pollen phase [PPP] in the previous year before treatment [2016]), initiation of SLIT (2017), 1st PPP during SLIT (2017), end of SLIT (2017) and 2nd PPP during follow-up (2018). In this study, PPP²⁵ was defined as the period of at least three consecutive days, when the total pollen count, instead of specifically *Artemisia annua* pollen count, was ≥ 300 total pollen/1000 mm² each day. Data of total pollen concentration were provided by Beijing Meteorological Bureau, which was reported as grain per 1000 mm².

Blood samples from all patients were collected at the initiation of treatment, 1st PPP and the end of treatment, prepared for immunologic analysis (Online Repository). *Artemisia annua* sIgE and total IgE (tIgE) were assessed at the beginning of treatment, the 16th week and the 32nd week.

Nasal secretion was collected from each patient at the initiation of treatment, 1st PPP and the end of treatment, and processed as previously described²⁶ (Online Repository).

2.3 | Clinical efficacy

Daily symptoms and medication usage were recorded throughout the 32-week treatment. The primary end point in the study was the total nasal symptom scores (TNSS), ranged from 0 to 12, including four-point scales of four main nasal symptoms, at the baseline, 1st PPP and 2nd PPP. Secondary, exploratory end points included changes in four-point scores for nasal symptoms and eye discomfort, daily medication score (DMS) and combined symptoms medication score (CSMS) (detailed description in Online Repository). The responsiveness of SLIT was defined according to the improvements of TNSS at the 2nd PPP in 2018, with $< 20\%$ classified as nonresponder and $> 20\%$ classified as responder.²⁷

2.4 | Safety assessment

Safety profile was assessed according to adverse events (AEs) recorded in daily cards. The grading system of local AEs (LAEs)¹⁶ and systemic AEs (SAEs)²⁸ were based on the World Allergy Organization (WAO) criteria (Online Repository).

2.5 | Flow cytometry

FACS Aria flow cytometry (BD Biosciences) was used to assess T-cell subsets in peripheral blood by phenotyping according to a standardized protocol (Online Repository).

2.6 | Local inflammatory mediators in nasal secretion

Inflammatory mediators in nasal secretions were measured using commercial ELISA kits, including ECP (CUSABIO, Wuhan, China), histamine and cystatin 1 (CST 1) (Cloud-Clone Corp.), LTB4 (R&D Systems) and total protein (Beyotime); and the levels of inflammatory mediators were expressed as ng or pg/mg protein.

2.7 | Statistical analysis

Statistical analyses were performed using the SPSS 19.0 software (IBM Corp.). Data were assessed for normality and equal variation, and results were expressed as mean $\pm$ standard deviation (SD). Mann-Whitney *U* test or Student *t* test was used to analyse the differences between variables. Receiver operating characteristic (ROC) curves were used to set a clinical marker for the efficacy of SLIT and evaluate its sensitivity and specificity. Differences were considered significant at the 95% confidence level. $P < .05$ was considered to be statistically significance.

3 | RESULTS

A total of 93 participants (mean age 37.00 ± 7.49 years, 49.3% female [$n = 46$], 50.7% male [$n = 47$]) were screened, of whom 71 were eligible and underwent baseline assessments and randomized to receive *A annua*-SLIT ($n = 47$) or placebo ($n = 24$). Both groups were comparable with respect to gender, ethnicity, history of AR, atopic status and co-morbidity of asthma, atopic dermatitis (Table S1). Based on pollen concentration, the pollen season of *A annua* occurred from August to September (Figure 1). After the 32-week treatment, 41 patients in the SLIT group and 20 patients in the placebo group completed the follow-up of the second pollen season (Figure S1).

The symptoms scores at the baseline (PPP of 2016) were comparable between both groups; however, SLIT significantly decreased the symptom scores for all symptoms, apart from watery eyes, at 1st and 2nd PPP, compared with placebo ($P < .001$, Figure 2). No significant intragroup difference was observed at either PPP in the placebo group.

Clinically, the nasal/ocular symptoms, apart from watery eyes, were significantly alleviated from baseline at the 1st and 2nd PPP in the SLIT group ($P < .001$, Figure 2). Although changes in nasal congestion and rhinorrhea were significantly different between the first

and second PPP ($P = .023$, $P = .020$, respectively) in the SLIT group, no intragroup differences were observed at these two time points for other nasal/ocular symptoms including sneezing, nasal itching, gritty eyes and watery eyes.

Total nasal symptom score and CSMS were comparable between both groups at baseline (PPP of 2016). SLIT significantly decreased TNSS to a greater level than placebo at both 1st and 2nd PPP ($P < .001$, Figure 3) and CSMS to a greater level than placebo at 2nd PPP ($P = .002$; Figure 3). In contrast, the DMS was not significantly different between the two groups at either baseline, 1st PPP or 2nd PPP (Figure 3B).

After 16-week preseasonal SLIT, TNSS in the SLIT group was significantly reduced from baseline at the 1st PPP (9.45 ± 1.68 vs 6.16 ± 2.27 , $P < .001$) and the 2nd PPP (9.45 ± 1.68 vs 5.59 ± 2.46 , $P < .001$; Figure 3A). The difference in TNSS was not significantly different between at the 1st PPP and the 2nd PPP (5.59 ± 2.46 vs 6.16 ± 2.27 , $P = .32$).

After 16-week preseasonal SLIT, CSMS in the SLIT group was not significantly decreased from baseline at the 1st PPP (4.74 ± 1.06 vs 3.32 ± 1.39 , $P = .072$), but was significantly decreased at the 2nd PPP (4.74 ± 1.06 vs 2.61 ± 1.43 , $P = .002$). The difference in CSMS was significantly different between the 1st PPP and the 2nd PPP (3.32 ± 1.39 vs 2.61 ± 1.43 , $P = .007$; Figure 3C).

After 32 weeks of the trial, significantly more patients in the placebo group developed sensitization to new allergen than patients in the SLIT group ($P = .008$), with 14 subjects (58.3%) in placebo group and 11 patients (23.4%) in SLIT group developing sensitization to new allergen (UniCAP $\geq$ ++).

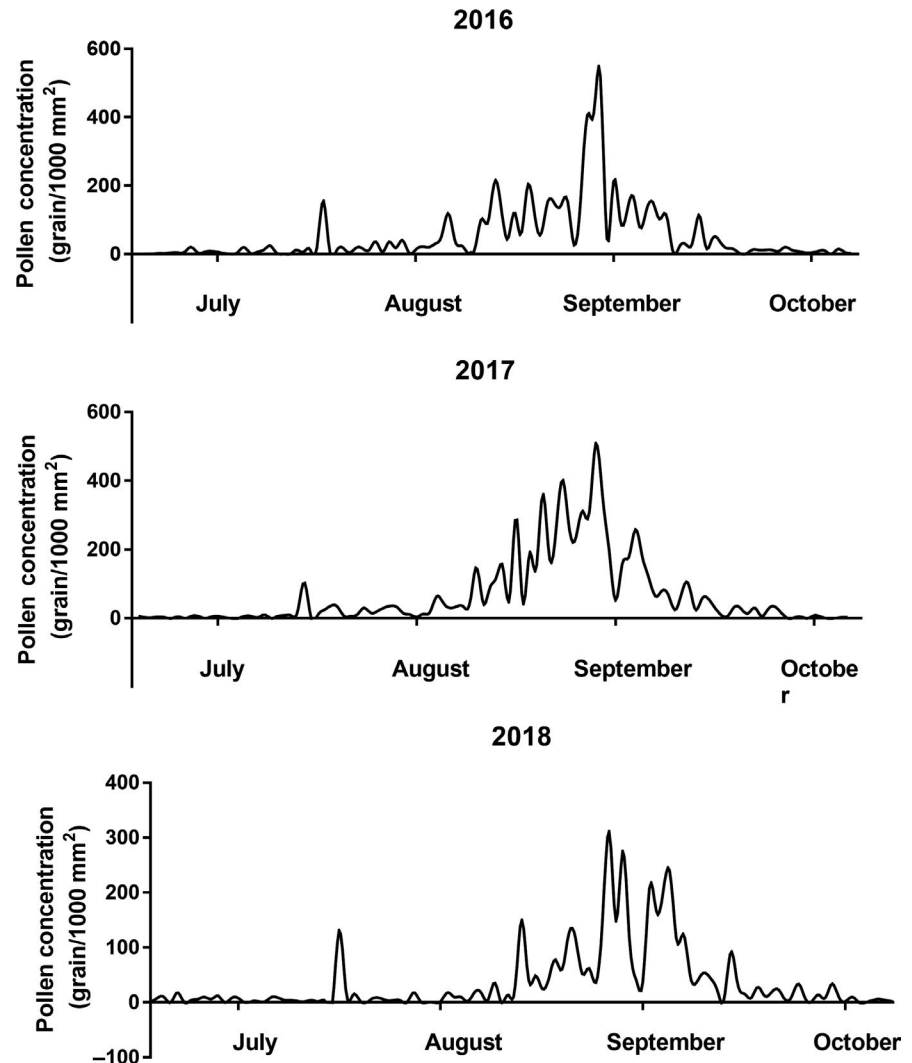
3.1 | Immunological evaluation

The levels of sIgE to *A annua* were comparable in the SLIT and placebo groups prior to treatment (40.33 ± 17.37 and 36.82 ± 15.99 KU/L, respectively). In the SLIT group, sIgE significantly increased from baseline following 16 weeks of preseasonal SLIT (from 40.33 ± 17.37 to 49.84 ± 25.42 , $P = .006$), but in the placebo group it not altered in the first 16 weeks (from 36.82 ± 15.99 to 35.10 ± 24.33 , $P = .539$). Following the next 16-week seasonal treatment, the levels of *A annua* sIgE were significantly increased at the end of 32nd week in both the SLIT and placebo-treated groups, compared with the end of the 16th week ($P = .0001$ and 0.005 , respectively).

Similarly, the level of tIgE increased significantly from the baseline level at initiation of the study to the end of the 16th week in SLIT group (from 182.9 ± 265.6 to 313.8 ± 325.5 , $P < .0001$), until it plateaued at the end of the treatment in the SLIT group. In contrast, the level of tIgE was not significantly altered from baseline over the entire course of treatment in the placebo group.

The ratio of sIgE/tIgE was assessed at the beginning of treatment, the 16th week and the 32nd week. The *A annua* sIgE/tIgE ratio was stable and comparable over the three time points (0.13 ± 0.09 , 0.18 ± 0.11 , 0.18 ± 0.13 , $P = .066$). Responsiveness to SLIT was

FIGURE 1 Pollen concentration in Beijing in 2016, 2017 and 2018



defined on basis of the TNSS improvements between baseline and 2nd PPP, and using this criterion, 78.7% of participants were found to be responsive and 21.3% nonresponsive. However, the responsive and nonresponsive groups were not significantly different with regard to baseline levels of Th2, Treg and Tr1 ($P = .17, 0.43$ and 0.89 , respectively) or the local expression of ECP, HA, ITB4 and CST1 ($P = .83, 0.51, 0.83$ and 0.083 , respectively).

Receiver operating characteristic curve analysis further demonstrated that the sIgE/tIgE ratio, with area under the curve (AUC) of 0.83 , was a significant predictor of responsiveness (Figure 6). According to the Youden index, when the sIgE/tIgE ratio was set to be 22% , the predictability was best. In contrast, Th2, Treg and Tr1 cells did not appear to be of significance in predicting responsiveness.

Analysis of the T-cell response in peripheral blood at the beginning of treatment, 16th week and 32nd week of SLIT indicated that Th1 cells were not significantly altered in either group (Figure 4A). However, after the 16-week preseasonal SLIT, the numbers of Th2 cells in the SLIT group decreased significantly from levels at the initiation of treatment (from 4.14 ± 2.14 to 2.90 ± 1.31 , $P = .0018$) and remained lower than in the placebo control group (Figure 4B). In contrast, nTreg cells increased significantly from pretreatment

levels (5.12 ± 1.67) after 16 weeks and 32 weeks in the SLIT group ($6.03 \pm 1.97, 6.22 \pm 2.13$, respectively; $P = .017$; Figure 4C). The numbers of Tr1 cells were also increased significantly from pretreatment levels after 16 weeks (from 0.92 ± 0.34 to 2.68 ± 1.45 , $P = .0006$; Figure 4D) and after 32 weeks (from 0.92 ± 0.34 to 1.32 ± 0.84 ; $P = .029$) in the SLIT group (Figure 4D). The levels of Th1, Th2, nTreg and Tr1 were not significantly altered over the course of 32 weeks in the placebo group (Figure 4A-D).

3.2 | Local inflammatory mediators

Analysis of the inflammatory mediators in nasal secretions demonstrated that the levels of ECP, histamine and LTB4 were not significantly different in the SLIT and placebo groups at the beginning of treatment, 1st peak pollen phase (PPP) and the end of treatment (Figure 5A,B,C). In contrast, although the local expression of CST1, a type 2 cysteine protease inhibitor, was also comparable between groups at the beginning of treatment (112.5 ± 93.66 in placebo group vs 73.07 ± 53.01 in SLIT group); this was found to be significantly increased in the SLIT compared with the placebo group, both

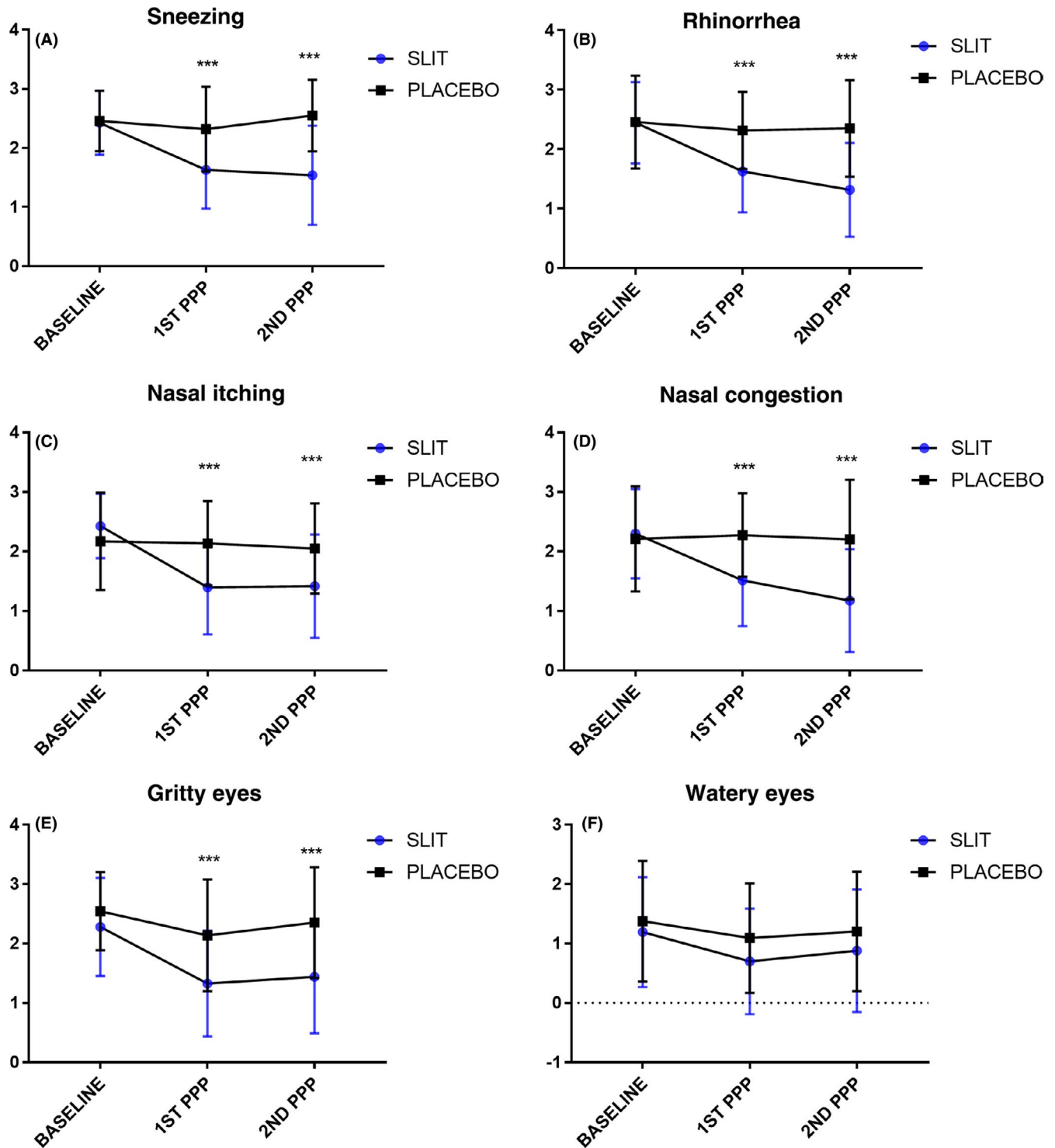


FIGURE 2 Comparison of symptom scores of sneezing (A), rhinorrhea (B), nasal itching (C), nasal congestion (D), gritty eyes (E) and watery eyes (F), between SLIT and placebo groups at baseline (2016, the peak pollen phase [PPP] of the year before treatment), 1st PPP and 2nd PPP. *** $P < .001$

at the 1st PPP (587.1 ± 319.2 vs 308.0 ± 153.3 , $P = .006$) and the end of treatment (535.8 ± 316.6 vs 286.5 ± 205.4 , $P = .009$; Figure 5D). Similarly, the levels of CST1 at the 1st PPP and the end of treatment were significantly different compared with the initiation of treatment in the SLIT group (587.1 ± 319.2 vs 73.07 ± 53.01 , $P < .0001$; 535.8 ± 316.6 vs 73.07 ± 53.01 , $P < .0001$).

3.3 | Safety

A total of 17/47 patients (36.2%) in the SLIT group experienced at least one local AE; such as numbness of tongue or mouth and mouth ulcer. All of the local AEs were Grade 1 and resolved with no treatment. Two patients in the SLIT group experienced a total of

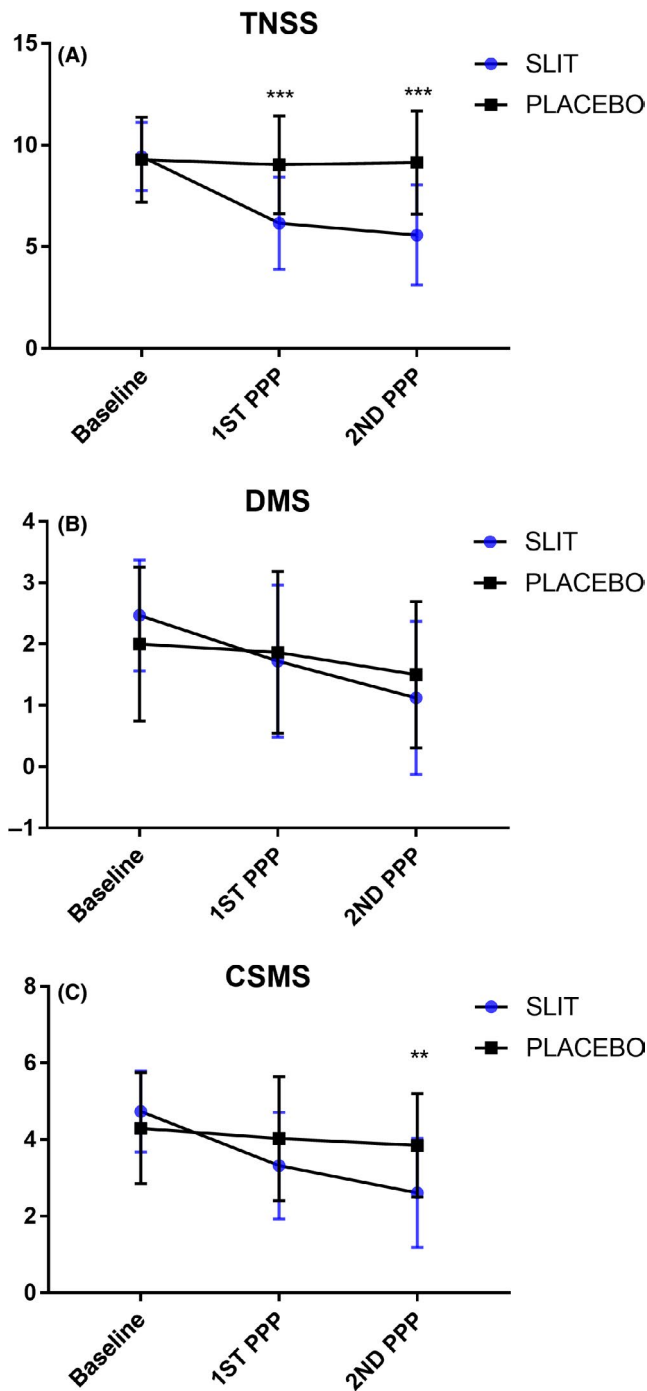


FIGURE 3 Comparison of combined symptom scores, TNSS (A), DMS (B) and CSMS (C), between SLIT and placebo groups at baseline, 1st PPP and 2nd PPP. Intergroup comparisons are shown in the figure. TNSS, total nasal symptoms scores; DMS, daily medication scores; CSMS, combined symptoms medication score. ** $P < .01$; *** $P < .001$

2 systemic reactions; 1 mild urticaria and flushing (Grade 1) and 1 asthma and chest tightness (Grade 3); both of which were moderate and resolved by use of an antihistamine. No serious AEs requiring epinephrine injection or caused discontinuation of trial were recorded.

4 | DISCUSSION

Artemisia annua is the most common aeroallergen in autumn responsible for SAR in northern China. However, to date *A annua* allergen has neither been available as a commercial AIT product nor has *A annua*-SLIT been investigated for clinical efficacy in a well-designed study. In this regard, the present randomized, double-blinded, placebo-controlled study is the first clinical trial to evaluate the clinical efficacy and immunological changes resulting from 32 weeks SLIT with *A annua* in SAR patients. The study demonstrated that SLIT led to significant improvements in nasal/ocular symptoms and CSMS, before and during the pollen season, over a total period of 32 weeks. Furthermore, the efficacy of SLIT with *A annua* persisted even after immunotherapy was stopped until 2nd season. Indeed, improvements in the individual symptom scores for rhinorrhea and nasal congestion, as well as TNSS at 2nd pollen season, were found to be even greater than those at 1st pollen season following 32-week SLIT. Also, according to WAO,²⁷ the clinical efficacy of *A annua*-SLIT was confirmed as there was a difference of at least 20% improvement between the clinical efficacies of SLIT and placebo groups.

Our findings for the reductions in symptom scores are in accordance with AIT trials with other allergens.⁸⁻¹¹ Similarly, our findings for the extended effects of 32-week SLIT into the following year are also in accordance with the findings of a recent trial of 2-year SLIT with Japanese cedar pollen allergen extract, which indicated that the efficacy of this treatment could last well into 3rd season.²⁰ Although Scadding and colleagues²⁹ were unable to demonstrate maintenance of clinical efficacy at 3-year follow-up after 2-years of SLIT with grass pollen extracts in patients with moderate to severe SAR, SLIT did nevertheless demonstrate sustained immunological tolerance demonstrated for secondary outcomes. We have additionally observed supporting evidence of efficacy in preventing the onset of new allergen sensitization. Although other studies have also provided evidence,^{30,31} this has been low-grade evidence; and consequently more robust, high-quality clinical trials are needed.

Apart from satisfactory clinical efficacy, our results also revealed a favourable safety profile. Although 36.2% patients in the SLIT group experienced at least one local AE, such as oral/facial pruritus, mouth oedema and throat irritation, these AEs were mild to moderate, occurred mostly during the accumulating phase, and did not require rescue medication. Our findings are consistent with other studies, which have also reported incidence of isolated oral and mild 0.35-5.2 AEs per patient,³² mostly during the first week of AIT.³³ Indeed, our results are consistent with previous reviews^{14,34} and RCTs.^{8,18,35}

In recent years, the efficacy and responsiveness to AIT in allergic rhinoconjunctivitis and asthma patients have aroused interest,²¹ particularly in identifying reliable, reproducible biomarkers predicting responsiveness. The latest EAACI position paper²¹ documented seven domains of potential biomarkers. Zeng and colleagues³⁶ reported that the levels of sIgE and sIgG4 increased in the first year of SLIT and reached a plateau thereafter. Similarly, Suarez-Fueyo and

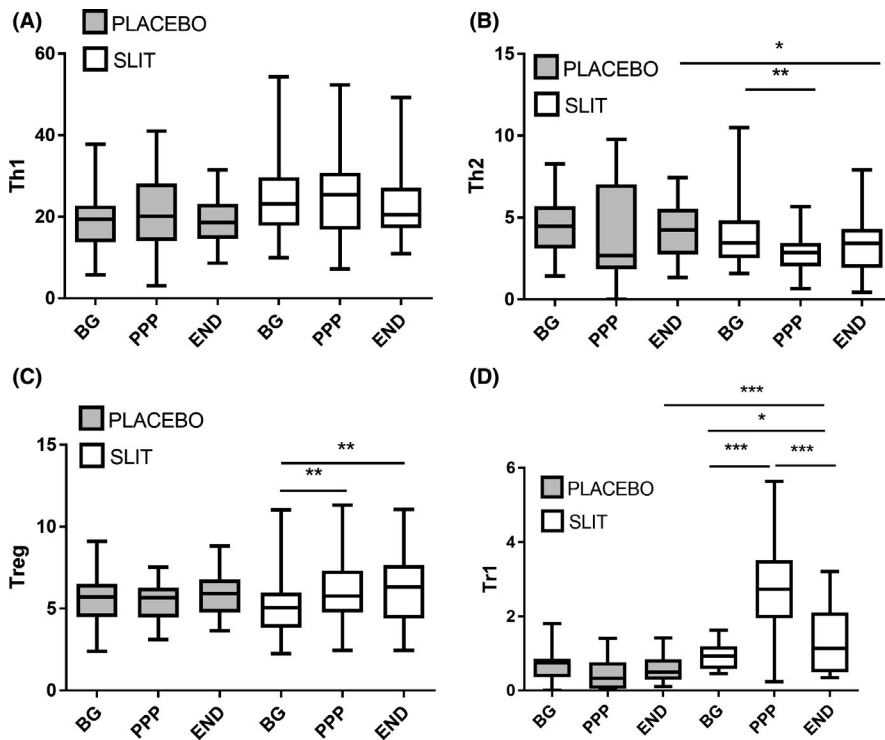


FIGURE 4 Comparison of the level of Th1 (A), Th2 (B), Treg (C) and Tr1 (D) cells between SLIT and placebo groups at the beginning of treatment, 1st peak pollen phase (PPP) and the end of treatment. BG, the beginning of treatment. * $P < .05$; ** $P < .01$; *** $P < .001$

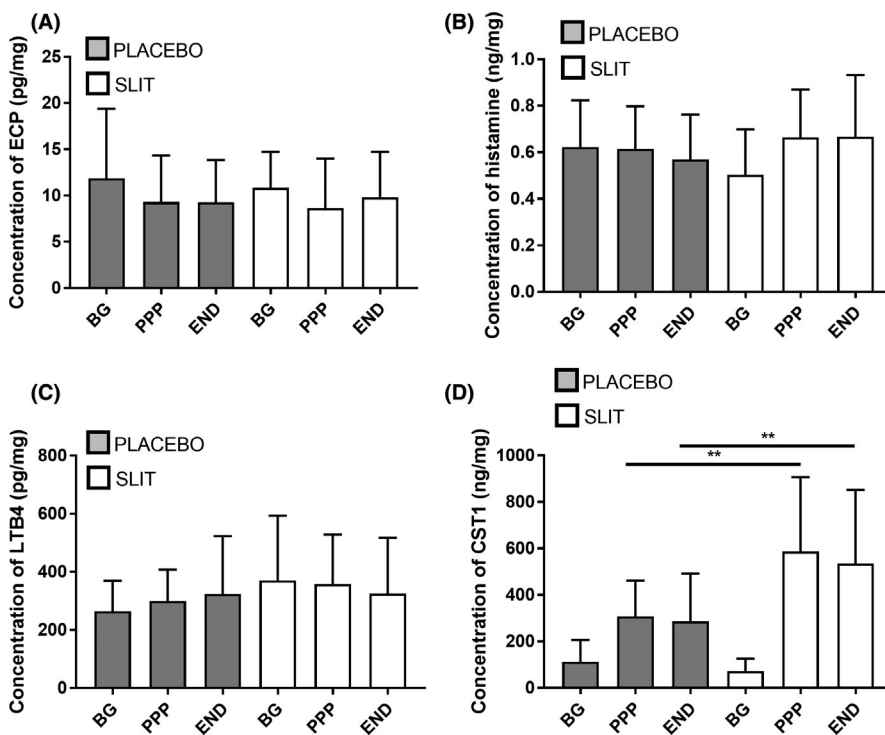


FIGURE 5 Comparison of the level of local inflammatory mediators between SLIT and placebo groups at the beginning of treatment, 1st peak pollen phase (PPP) and the end of treatment. BG, the beginning of treatment. * $P < .05$; ** $P < .01$; *** $P < .001$

colleagues³⁷ showed that grass pollen SLIT led to increased sIgE and sIgG4, together with Th2 responses. They suggested that increased sIgE level during the first month was indicative of an sIgG4 response in patients. Another study reported that baseline measures of basophil reactivity and sIgE/tIgE ratio might help identify patients' responsiveness to therapy.³⁸ Tosca and the colleagues³⁹ found that paediatric patients with sIgE > 10 kU/L were more likely to perceive AIT efficacy and therefore suggested that sIgE assessment might

be a potential marker for responders. Di Lorenzo and colleagues⁴⁰ observed the prediction value of sIgE/tIgE ratio (16.2%) in AIT for monosensitized patients. However, no consensus has been reached. Our finding showed that the level of sIgE was progressively elevated during 32 weeks of SLIT with *A annua* is consistent with previous studies. Furthermore, ROC curve analysis indicated that the sIgE/tIgE ratio (>22%), whose AUC was 0.83, was a potentially good marker of responsiveness.

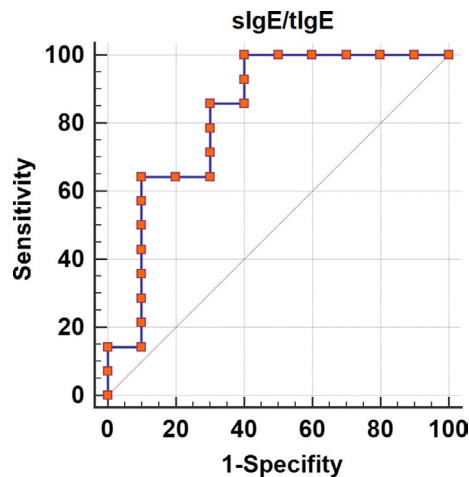


FIGURE 6 Receiver operating characteristic (ROC) curve of the predictive model of SLIT responsiveness

It is likely that the immunological mechanisms of SLIT are different to those of SCIT.^{41,42} However, whilst one recent study has shown a downregulation of Th2 immune responses followed by induction of Tregs after SLIT,³⁷ other two studies have failed to demonstrate any SLIT-mediated deviation of Th2/Th1 polarization and Treg induction.^{43,44} In accordance with previous findings,³⁷ the present study demonstrated downregulation of Th2 immune response followed by induction of Tregs, after SLIT with *A annua* allergen. Although Th1 cells were not significantly altered over the course of the entire study, the Tr1 cells increased after preseasonal SLIT (from 0.92 ± 0.34 to 2.68 ± 1.45). This may be of particular relevance because Tr1 cells produce high levels of IL-10 and TGF- β , and low levels of IFN- γ and IL-2 in the absence of IL-4⁴⁵ and therefore modulate several facets of allergic inflammation.^{46,47} The upregulation of Tr1 in the present study indicates modification of the systemic immune system during the preseasonal treatment.

In contrast to the changes in T-cell subtypes, no significant differences were noted in the levels of ECP, histamine and LTB₄ between SLIT and placebo group. It may be a consequence of the relatively small sample size, and thus, larger studies are needed in the future. However, our study has demonstrated that the level of local CST1, a member of type 2 cystatin proteins superfamily, was significantly increased during SLIT, compared with placebo treatment. In recent years, CST1 has been shown to be associated with allergic disease, such as respiratory allergy⁴⁸ and SAR.⁴⁹ In particular, there is some evidence that CST1 is upregulated in the nasal mucosa of patients with seasonal AR during the pollen season.⁴⁹ Similarly, evidence from one very recent study suggests that human CST1 can inhibit specific pollen allergen-induced disruption of epithelial tight junction barrier in a human nasal cell line in vitro and also suppress specific pollen allergen-induced nasal symptoms and tight junction disruption in a transgenic mouse model of AR expressing human CST1.⁵⁰ Based on the findings of these studies, it is reasonable to speculate that continuous exposure to specific allergen during AIT could elevate the level of CST1, which would in turn create a positive loop in attenuating the allergen-induced structural changes at the nasal mucosal

level. In this sense, CST1 might be considered a marker of the protective effects of AIT in SAR patients; however, the role of CST1 in AIT and its relationship with eosinophils in AR is presently unclear and needs to be evaluated in future studies.

However, our findings are limited in some ways. Firstly, total pollen counts were recorded instead of specifically *A annua* pollen counts in the study. Nevertheless, *A annua* was found to be the major outdoor aeroallergen in autumn in northern China responsible for hay fever or asthma, and therefore, it is likely that these findings accurately reflect the effects of *A annua*-SLIT noted in patients sensitized to this allergen. Secondly, the course of treatment and the follow-up period were relatively short compared with many other studies, which have traditionally investigated the effects of AIT over longer periods in patients with allergic disease and therefore do not give any indication of the long-term effects of *A annua*-SLIT, either in China or worldwide in regions where *A annua* pollinosis is prevalent. Thus, further studies specifically addressing these limitations are required to confirm the findings of the present study in the future.

In conclusion, 32 weeks of SLIT with *A annua* is an efficacious and safe treatment option in patients with *A annua*-induced SAR, and the effects of therapy are sustained into the following pollen season. The mechanisms underlying the effects of SLIT are systemic and present as downregulation of Th2 immune responses followed by the induction of Tregs.

CONFLICT OF INTEREST

All authors declare no financial or commercial conflicts of interest.

AUTHORS' CONTRIBUTIONS

YH, CW, YO, YZ, LX and HL contributed to acquisition of data. XC and YW performed FACS. HL and LZ provided input to study design. All authors were responsible for data analysis and preparation of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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93 patients were assessed for eligibility

22 were not eligible for inclusion in the study

71 underwent baseline assessments and randomization

47 were assigned to SLIT

47 completed 32-week assessment

41 completed yr-2 assessments
5 lost to follow-up
1 moved away

24 were assigned to placebo

24 completed 32-week assessment

20 completed yr-2 assessments
4 lost to follow-up

METHODS

Exclusion criteria

Exclusion criteria included previous immunotherapy with pollen extracts, a clinical history of perennial AR or asthma, any nasal condition that could confound the results of the study (chronic rhinitis, chronic rhinosinusitis with/without polyps), non-compliance with study protocol or treatment, pregnancy, sexually active women with childbearing potential, and patients with immunologic suppression diseases.

Sample estimation

The sample size was estimated by power statistics using TNSS as the primary outcome measure. Based on data from the previous phase II clinical trial, the estimated mean difference between groups was 2.2. Using a 2:1 group design, 37 patients in the SLIT group and 19 patients in the placebo group were required to give a 5% risk of type 1 error and 10% type 2 error. Considering a drop-out rate of 20%, we recruited 71 participants, with 47 subjects in SLIT group and 24 subjects in placebo group.

Randomisation and Blinding

Randomisation was carried out according to a sequence generated using a computer random number generator. The treatment allocation was concealed by the randomisation sequence and all patients and investigators were blinded to the study treatments throughout the entire study. Data were unblinded 1 year after the completion of the trial.

Sublingual Immunotherapy protocol

The 32-week study protocol involved a “5-week” up-dosing (1.25BU to 2400BU) and a “27-week” maintenance period (2400 BU) with daily sublingual drops, which were provided by Zhejiang Wolwo Bio-Pharmaceutical Co., Ltd., China, as five vials of specific *Artemisia annua* extracts with concentrations ranging from 25 to 16,000 BU/ml. The allergen extract used for immunotherapy was glycerinated allergen extract of *Artemisia annua*, while the placebo drops were physically identical to active medication, but without allergenic components. The main allergenic components of the extracts were *Art an1* and *Art an3*. Drops were administered

sublingually for 1 minute before swallowing.

Clinical endpoints

The primary endpoint was the total nasal symptom scores (TNSS), which was calculated as the sum of scores for nasal congestion, discharge, itching and sneezing scored on a 4-point scale of 0-3, and ranging from 0-12. Secondary, exploratory endpoints included changes in 4-point scores for nasal symptoms and eye discomfort, daily medication score (DMS), and combined symptoms medication score (CSMS). The DMS was calculated according to the total daily intake of medication scored on a 3-point scale; with 1= oral antihistamines; 2= intranasal corticosteroids; 3= oral corticosteroids. CSMS was assessed as the combination score of the average score for six symptoms (rhinorrhea, nasal congestion, nasal itching, sneezing, watery eyes and gritty eyes) and the DMS(1).

Blood sample preparation

An aliquot of whole blood from each sample was used for analysis by flow cytometry, and the remaining blood sample was centrifuged within 2 hours of collection. The resulting plasma samples were stored at -80 °C for analysis of inflammatory mediators at a later stage.

Nasal secretion preparation and Elisa assay

Briefly, the nasal secretion was collected by inserting a sponge pack in the common meatus for 5 minutes; and the quantity of nasal secretion calculated as the difference in the weight of the sponge pack before and after collection of the secretion. One mL of 0.9% sodium chloride solution was added to each sponge pack, and the packs incubated at 4°C for 2 hours prior to centrifugation at 1500 rpm for 8 minutes at 4°C. At the end of centrifugation, the supernatants were separated and stored at -80°C for analysis of eosinophil cationic protein (ECP), leukotriene B4 (LTB4), histamine and Cystatin 1 (CST1) at a later stage.

The concentration of ECP was determined according to the manufacturer's instructions using commercial Elisa kits, obtained from Cusabio Life Sciences, Wuhan, China. Similarly, the concentrations of histamine and CST1 were measured according to the manufacturer's instructions using Elisa kits obtained from

Cloud-Clone Corp., Wuhan, China, and the concentrations of LTB₄ were detected by Elisa kits obtained from R&D Systems, Minneapolis, Minn. The color intensity of Elisa reactions was measured using a Spectramax Plus reader (Molecular Devices, San Jose, CA, USA). The samples were also analyzed for protein concentration using BCA protein assay kit (Beyotime, Beijing, China), and the concentrations of ECP, histamine, CST1 and LTB₄ were expressed as ng or pg/mg protein. The results were excluded for samples with concentrations lower than the detection limit of the Spectramax Plus reader.

Flow cytometry

The cells were labelled with specific monoclonal antibodies (BD Biosciences, NJ, USA); including anti-CD4-PE Cy7, anti-CD25-APC, anti-CD127-PE, anti-IL-4-APC, anti-IL-10-PE, and anti-IFN- γ -FITC. T cell subsets were stained for detailed phenotypic analysis as follows: (1) T helper (Th) 1 cells: IFN- γ +IL-4-CD4⁺ T cells; (2) Th2 cells: IFN- γ -IL-4+CD4⁺ T cells; (3) Regulatory T (nTreg) cells: CD4+CD25+CD127- T cells; (4) Type 1 regulatory T (Tr1) cells: IL-10+IL-4-CD4⁺ T cells.

Safety assessment

Local adverse effects involved pruritus/swelling of mouth, tongue or lip, throat irritation, nausea, abdominal pain, vomiting, diarrhea, heartburn and uvular edema. Grade 1 is mild, not troublesome, no symptomatic treatment required and no discontinuation of SLIT. Grade 2 is moderate, troublesome, or required treatment, but no discontinuation of SLIT. Grade 3 is severe, troublesome, required treatment and also discontinuation of SLIT(2).

Systemic adverse effects were graded based on the proposed modification of the 2010 World Allergy Organization grading system(3), from Grade 1 to 5. Grade 1 included symptoms from one organ (cutaneous, conjunctival, upper respiratory, or other nonspecific organ systems). Grade 2 included symptoms from ≥ 2 organ symptoms listed in Grade 1. Grade 3 involved asthma responded to medicine or gastrointestinal or uterine cramps. Grade 4 included asthma not responding to medicine. Grade 5 indicated respiratory or cardiovascular failure.

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Figures and Tables

Table S1. Demographics and baseline disease characteristics of participants

Characteristics	SLIT	Placebo	<i>P</i> -value
Total subjects, n	47	24	-
Gender (%), male/female	23(48.9)/24(51.1)	13(54.2)/11(45.8)	0.8
Age (years)	38.70±7.75	33.67±5.77	0.07
Ethnic group (%), Han/ not Han	44(93.6)/3(6.4)	22(91.7)/2(8.3)	1.00
History of AR	9.51±6.63	8.46±5.06	0.5
History of smoking (%), yes/no	4(8.5)/43(91.5)	4(16.7)/20(83.3)	0.44
Allergic conjunctivitis (%), yes/no	40(85.1)/7(14.9)	24(100.0)/0(0.0)	0.09
Asthma (%), yes/no	9(19.1)/38(80.9)	4(16.7)/20(83.3)	1.00
Atopic dermatitis (%), yes/no	4(8.5)/43(91.5)	3(12.5)/21(87.5)	0.68
Food allergy (%), yes/no	5(10.6)/42(89.4)	2(8.3)/22(91.7)	1.00
Drug allergy (%), yes/no	8(17.0)/39(83.0)	4(16.7)/20(83.3)	1.00
Other allergic diseases (%), yes/no	42(89.4)/5(10.6)	24(100.0)/0(0.0)	0.16

AR, allergic rhinitis

Figure S1. Flow diagram illustrating the numbers of subjects screened, randomized to treatment, and evaluated during the study.