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Rhinitis, Sinusitis, and Upper Airway Disease

Exhaled Volatile Organic Compounds Identify Allergic Patients Among Individuals With Chronic Rhinitis

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ABSTRACT

Background: The nasal allergen challenge (NAC) is the gold standard to diagnose allergic rhinitis (AR) and local allergic rhinitis (LAR), but its clinical use remains limited. We investigated whether volatile organic compounds (VOC) in exhaled breath could identify allergic individuals among patients with chronic rhinitis.

Methods: Exhaled breath samples were collected before and 24 h after NAC in participants with AR, LAR, non-allergic rhinitis (NAR), and healthy control (HC). Samples were analyzed using gas chromatography–mass spectrometry, focusing on saturated hydrocarbons. Associations with fractional exhaled nitric oxide (FeNO) and blood eosinophils were also assessed.

Results: We included 28 AR, 31 LAR, 29 NAR, and 14 HC individuals, divided into training and validation sets. A predictive model based on two VOCs (decane and nonadecane) discriminated allergic (AR + LAR) from non-allergic (NAR + HC) subjects (AUC 0.721, 95% CI: 0.506–0.936; permutation test $p = 0.009$), with moderate performance in the validation set (AUC 0.760, 95% CI: 0.585–0.935; 76.5% sensitivity, 69.2% specificity). Similarly, these two VOCs moderately differentiated LAR and NAR patients (AUC 0.737, 95% CI: 0.545–0.929; permutation test $p = 0.011$). At baseline, decane levels were higher in LAR than HC subjects ($p = 0.007$), nonadecane levels were higher in LAR than NAR individuals ($p = 0.026$), and decane, styrene, and nonanal levels were higher in patients with FeNO ≥ 25 ppb (all $p \leq 0.016$). No associations were observed between VOCs and blood eosinophils. NAC induced a significant reduction in nonadecane in allergic patients ($p = 0.012$), but not in non-allergic subjects.

Conclusion: Exhaled decane and nonadecane might serve as non-invasive biomarkers of allergic inflammation in rhinitis patients, potentially supporting NAC indication. Elevated exhaled nonanal, decane, and styrene may reflect T2 airway inflammation. These exploratory findings require validation in larger, multicenter cohorts with accuracy-improving potential.

Abbreviations: AA, *Alternaria alternata*; AIT, allergen immunotherapy; AR, allergic rhinitis; AUC, area under the curve; BEC, blood eosinophil count; DP, *Dermatophagoides pteronyssinus*; FeNO, fractional exhaled nitric oxide; GC, gas chromatography; HC, healthy control; LAR, local allergic rhinitis; LASSO, least absolute shrinkage and selection operator; LR, logistic regression; MS, mass spectrometry; NAC, nasal allergen challenge; NAC-M, nasal challenge with multiple allergens; NAC-S, nasal challenge with single allergen; NAR, non-allergic rhinitis; OE, *Olea europaea*; PJ, *Parietaria judaica*; PP, *Phleum pratense*; ROC, receiver operating characteristics; ROS, reactive oxygen species; sIgE, allergen-specific IgE; SPT, skin prick test; TD-GC/q-MS, thermal desorption system coupled to gas chromatography-single quadruple mass spectrometry; TDT, thermal desorption tube; VOC, volatile organic compound.

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1 | Introduction

Allergic rhinitis (AR) affects 25% of individuals in Western countries and is associated with a significant decrease in quality of life [1]. AR diagnosis requires the positivity of atopy tests (skin prick test (SPT) and/or serum allergen-specific (s)IgE), along with the documentation of the impact of allergen exposure on rhinitis symptoms [2]. The latter is straightforward for seasonal allergens, but it is often challenging for perennial allergens or polysensitized patients [3]. In this regard, a nasal allergen challenge (NAC) can help clarify the relevance of positive atopy tests [3]. Interestingly, some non-atopic patients also test positive to the NAC, and this scenario is referred to as local allergic rhinitis (LAR) [2]. LAR is an independent disease phenotype [4] affecting 16%–25% of non-atopic patients with rhinitis [5, 6]. Both AR and LAR are IgE-mediated conditions [7] naturally evolving toward the aggravation and the onset of asthma [1, 4]. On the other hand, allergen immunotherapy (AIT) controls nasal symptoms and provides long-term benefits after discontinuation in both rhinitis phenotypes [8–11]. Moreover, AIT prevents the onset of asthma and new allergic sensitizations [10, 11]. Therefore, the early identification of rhinitis patients benefitting from AIT is essential to establish successful preventive strategies.

Despite representing the gold standard for the allergic triggers of rhinitis, the NAC also has limitations (time-consuming nature, need for patient collaboration, shortage of allergen extracts for provocation, etc.) [12]. Thus, there is a growing interest in novel tools to identify allergic patients in the clinic. Volatile organic compounds (VOCs) in exhaled breath have emerged as promising biomarkers in respiratory [13] and extra-respiratory [14] diseases. Exhaled VOCs reflect metabolic processes including reactive oxygen species (ROS) generation and lipid peroxidation in airway resident cells or microbiota and differ markedly between homeostatic and inflammatory states [14]. Fractional exhaled nitric oxide (FeNO), a routinely used biomarker for T2 airway inflammation, is a good example of the utility of breath-based diagnostics. Thanks to novel gas chromatography–mass spectrometry methods, it is now possible to perform a detailed determination of VOCs in biological samples, including urine and exhaled breath [13–15]. Of note, these samples are extremely easy to collect even in uncooperative patients [16]. Nevertheless, no study has yet investigated the capacity of exhaled VOCs to differentiate allergic from non-allergic subjects, as defined by NAC result [2].

In this study, we recruited patients with perennial rhinitis and allocated them into AR, LAR, or non-allergic rhinitis (NAR) groups. Healthy controls (HC) were also included. Exhaled breath samples were collected before and 24 h after an NAC. We focused on saturated hydrocarbons, a VOC class previously associated with asthma (specifically decane [17, 18]), and on VOCs linked to eosinophilic asthma (specifically acetone, styrene, and nonanal [19]). As the primary objective, baseline samples were used to identify a VOC signature capable of discriminating NAC-confirmed allergic individuals. As a secondary aim, we assessed correlations between VOCs and general T2 biomarkers (non-specific for allergic phenotype) and explored whether VOC profiles changed 24 h post-NAC.

2 | Methods

2.1 | Study Individuals

Rhinitis patients aged ≥14 years with perennial nasal symptoms for ≥2 years were prospectively recruited from the Allergy Unit of Hospital Regional Universitario de Malaga between January and December 2023. All participants underwent SPT, serum sIgE quantification, and standardized NAC (see details below). Based on the diagnostic work-up, individuals were assigned to one of the following groups until the predefined sample size was reached:

- AR: positive SPT, serum sIgE, and NAC to a single perennial allergen (*Dermatophagoides pteronyssinus* (DP), *Alternaria alternata* (AA) OR *Parietaria judaica* (PJ)); negative SPT and serum sIgE to all other allergens; and negative NAC to *Phleum pratense* (PP) and *Olea europaea* (OE). Only monoallergic patients were included. PJ behaves as a perennial allergen in Mediterranean coastal areas.
- LAR: negative SPT and serum sIgE to all tested allergens; positive NAC to a single perennial allergen (DP, AA, or PJ); and negative NAC with PP and OE. Only monoallergic patients were included.
- NAR: negative SPT and serum sIgE with all allergens and negative NAC with DP, AA, PJ, PP, and OE.

Individuals with regular exposure to furry animals also underwent NAC with cat and/or dog dander extracts, as appropriate. Moreover, patients' companions were recruited as HC if they reported no nasal or bronchial symptoms and showed negative results for SPT (all allergens), serum sIgE (all allergens), and NAC (DP, AA, PJ, PP, and OE).

Exclusion criteria included chronic rhinosinusitis, anatomical alterations preventing NAC interpretation (assessed by a nasal endoscopy) [20], severe and/or uncontrolled oncologic, metabolic, heart, inflammatory, infectious or systemic diseases, positive NAC to cat or dog, and pregnancy. All participants provided written informed consent prior to inclusion. The study protocol was approved by the Local Ethics Committee of Malaga and conducted according to the guidelines of the Declaration of Helsinki.

Asthma symptoms were not considered an exclusion criterion for patients, except in subjects with severe asthma, in whom NAC is contraindicated. However, a formal guideline-based confirmation of asthma [21] was not performed.

2.2 | Clinical and Environmental Data

Upon inclusion, all participants were interviewed regarding their lifestyle habits (smoking habit, fast food and alcohol consumption, and physical activity) and environmental exposures (occupation, pets, indoor dampness, residential location, proximity to high-traffic road, and distance from the sea) that can affect the exhaled volatilome [13, 17, 22]. Rhinitis severity was graded according to modified ARIA classification [23].

2.3 | Nasal Allergen Challenge

Two validated NAC protocols were performed with distinct objectives. For diagnostic classification, a multiple-allergen NAC (NAC-M) was conducted [24]. Standardized extracts of *DP (*100 HEP/mL), *AA, PJ, GP, OE, cat, and dog dander (all 30 HEP/mL*; Laboratorios LetiPharma, Spain) were applied bilaterally (100 μL/nostril) onto the head of the lower turbinate using a micropipette at 15-min intervals. A maximum of five allergens were tested per session. The procedure was stopped upon a positive response (see monitoring in the Supplement), with additional visits (≥ 1-week interval) scheduled to complete the testing.

Subsequently, a single-allergen NAC (NAC-S) was conducted to analyze the effect of allergen exposure on exhaled biomarkers. AR and LAR patients underwent an NAC-S with the relevant allergen identified during the diagnostic work-up (DP, AA, or PJ) [20]. Participants in the NAR and HC groups also received an NAC-S with one of these three allergens assigned in comparable proportions across groups.

2.4 | Other Clinical Procedures

The Supplement elaborates the methodology for SPT, quantification of serum sIgE and blood eosinophil count (BEC), FeNO measurement, and lung function testing. FeNO values ≥ 25 ppb were considered indicative of T2 inflammation.

2.5 | Collection and Analysis of Exhaled Breath Samples

Exhaled breath samples were obtained from all participants immediately before and 24 h after NAC-S. Individuals breathed at tidal volume into 1-L Tedlar gas sampling bag to obtain the mixed expiratory breath portion [25]. The exhaled air was immediately transferred to thermal desorption tubes (TDT; Tenax TA/carbograph 5td, Markes International). Ambient air samples were collected from the testing room into TDT using an Easy-VOC syringe (Markes International) to control for environmental influence. The samples were analyzed using a thermal desorption system coupled to gas chromatography-single quadruple mass spectrometry (TD-GC/q-MS). Chemical standards (C7–C30 saturated alkanes standard and VOC calibration standard, Sigma-Aldrich) were also analyzed by TD-GC/q-MS. Raw data from mass spectrometry were preprocessed using an open-source workflow previously detailed [25].

2.6 | Predictive Model of Allergic Respiratory Disease

A predictive model (Model I) was built to discriminate allergic (AR and LAR) from non-allergic (NAR and HC) individuals using the matrix of relative intensities of *m/z* signals obtained from the pre-NAC samples. Only features potentially related to saturated hydrocarbons were selected (those with *m/z* values compatible with linear alkanes, and/or showing characteristic linear alkane ion fragments (*m/z* = 57, 43 and 71)). The study population was randomly divided into training and validation sets (70% and 30% of subjects, respectively) (Figure S1) using a stratified randomization approach implemented with the *splitstackshape* package in R (version 4.0.5.).

Data modeling and validation were performed in R version 3.6.1, following the previously published protocol [25]. Before model construction, an unsupervised principal component analysis (PCA; *FactoMineR* package) [26] was conducted to assess natural group separation besides identifying potential external biases and outliers. The model was generated by least absolute shrinkage and selection operator (LASSO) and logistic regression (LR) (LASSO+LR) using the *glmnet* package (version 3.0–1) [27]. The training set served as a basis for model construction. Model development comprised two steps: parameter optimization and model construction. The regularization parameter λ was optimized via 5-fold cross-validation. The model performance was checked by two approaches: 5-fold cross-validation on the training set and additional testing on the validation set. NAC results represented the gold standard for comparisons, and model performance was assessed by calculating the area under receiver operating characteristics (ROC) curves (AUC), sensitivity, specificity, balanced accuracy, and positive and negative predictive values (PPV and NPV, respectively). ROC curves, AUCs, and their 95% confidence intervals (CI) were obtained using the *pROC* package [28]. The cut-off value was determined according to the case–control ratio in the training set. The R code for the main functions used for model construction and validation is provided in Figure S2. Moreover, the model significance was assessed by permutation test (1000 permutations) on the training set. P-values < 0.05 were considered statistically significant.

As an exploratory analysis, an additional model (Model IB) based on saturated hydrocarbons in pre-NAC samples was generated to differentiate LAR and NAR subjects. Model IB was built using LASSO+LR and evaluated by 5-fold cross-validation together with a permutation test.

Finally, the incremental diagnostic value of exhaled VOCs over established T2 biomarkers was assessed. For this purpose, a base model (FeNO+BEC) (Model II) and a combined model (FeNO+BEC + VOCs) (Model III) were generated to compare their predictive performances and estimate ΔAUC ($\Delta AUC = AUC_{Model\ III} - AUC_{Model\ II}$). Model II and III followed the same construction and validation protocol as Model I.

2.7 | Statistical Analysis

Based on previous exploratory studies demonstrating significant discriminatory capacity for VOCs [29], we estimated a minimum sample size of 100 individuals to ensure adequate representation of each rhinitis phenotype after splitting the cohort into training and validation sets. Statistical analysis was conducted using R software (version 4.0.5.). Continuous variables were expressed as mean ± standard deviation and categorical variables as frequencies and percentages. Normality of data distribution was assessed by Shapiro–Wilk test or Lilliefors tests (*nortest* package) [30]. Between-group comparisons were performed by Fisher's exact test or chi-square test for categorical variables and Student's *t*-test or ANOVA for continuous variables with normal

distribution. For non-normally distributed continuous data, Mann–Whitney U or Kruskal-Wallis tests were applied. When appropriate, *post hoc* analyses were conducted with Bonferroni correction for multiple comparisons. Paired data were analyzed using the paired *t*-test or Wilcoxon signed-rank tests, as appropriate. Correlations between continuous variables were evaluated using Pearson's correlation coefficient. A p-value <0.05 was considered statistically significant.

3 | Results

3.1 | General Characteristics of Study Participants

A total of 102 individuals were enrolled (65.7% females, mean age 38.05 ± 14.70 years), including 14 HC, 28 AR, 31 LAR, and 29 NAR subjects (Figure S3). Group comparisons are summarized in Table 1. The age of patients differed significantly among rhinitis phenotypes ($p=0.021$), with AR individuals being younger than NAR subjects ($p=0.005$). Most patients suffered from persistent rhinitis of moderate severity, and asthma symptoms were present in 21.4%, 12.9%, and 6.9% of AR, LAR, and NAR subjects, respectively. Both the allergic and non-allergic groups displayed comparable heterogeneity in T2 biomarker levels. Serum total IgE levels displayed significant differences across study groups ($p=0.008$), with higher concentrations in AR (212.8 IU/mL) compared to patients with LAR (80.0 IU/mL, $p=0.013$) and NAR (87.2 IU/mL, $p=0.023$). No significant differences were observed among groups in sex, body mass index, duration and persistence of rhinitis, asthma symptoms, FEV1, BEC, or FeNO values. Allergen sensitization profiles were comparable between AR and LAR individuals.

3.2 | Environmental Exposures and Lifestyle Habits

Signs of dampness at home were more frequently reported by allergic individuals than by non-allergic subjects ($p=0.013$), but there were no significant differences across the three groups of patients ($p=0.073$). Water leaks differed significantly among rhinitis phenotypes, being more frequent in LAR than in AR or NAR patients ($p=0.013$). Smoking habit, other lifestyle parameters, and household environmental features were comparable across study groups (Table 2). No significant association was found between city/town of residence and rhinitis phenotypes ($p=0.755$) (Figure S4).

3.3 | Primary Objective

3.3.1 | Predictive Model of Respiratory Allergic Disease Based on Exhaled Breath Biomarkers

The study population was randomized into training ($n=72$) and validation ($n=30$) sets using a stratified randomization approach (Figure S1), with no relevant differences between sets in phenotype distribution (AR/LAR/NAR/HC) or in the proportion of participants reporting asthma symptoms (Figure S5). Model I used LASSO+LR to discriminate allergic (AR + LAR) from non-allergic (NAR + HC) individuals by analyzing saturated hydrocarbons in pre-NAC samples. The PCA-based exploratory analysis conducted before model construction revealed no clustering according to phenotype (AR/LAR/NAR/HC), season of sample collection, or analytical batch/date of analysis (Figure S6 and Figure S7). In the training set, the model achieved a 5-fold cross-validated AUC of 0.721 (95% CI: 0.506–0.936; permutation test $p=0.009$). In the validation set, the AUC was 0.760 (95% CI: 0.585–0.935), with a sensitivity of 76.5% and a specificity of 69.2%. Decane and nonadecane were identified as the most relevant discriminative VOCs (Figure 1).

Model IB allowed discrimination between LAR and NAR patients with statistical significance (AUC 0.737, 95% CI: 0.545–0.929; permutation test $p=0.011$), again identifying decane and nonadecane as the main discriminative variables (Figure S8).

The FeNO-/BEC-based model (Model II) showed poorer performance than the combined FeNO/BEC/VOC model (Model III) in both the training ($\Delta AUC=0.120$; Model II: AUC 0.582 [95% CI:0.370–0.794], permutation test $p=0.163$; Model III: AUC 0.702 [95% CI:0.566–0.839], permutation test $p=0.022$) and validation ($\Delta AUC=0.221$; Model II: AUC 0.565 [95% CI:0.350–0.781]; Model III: AUC 0.787 [95% CI:0.624–0.951]) sets (Figure S9 and Figure S10). Models I and III showed similar overall performances (Figure 1), while sensitivity in the validation set for discriminating LAR patients from non-allergic subjects was higher for Models I and III than for Model II (77.8% vs. 33.3%) (Figure S11).

3.4 | Comparison of Predictive VOCs Across Study Groups at Baseline

At baseline, the relative abundance of decane and nonadecane differed significantly across study groups ($p=0.0093$ and $p=0.0092$, respectively; Figure 2A). Both compounds showed the highest levels in LAR patients. Decane levels were significantly higher in LAR compared with HC ($p=0.007$), while nonadecane was significantly elevated in LAR compared with NAR patients ($p=0.026$). Similar results were obtained when subjects with asthma symptoms (Figure S12A) and participants living in households with dampness (Figure S13A) or water leaks (Figure S14A) were excluded from the analysis. Additionally, the sensitizing allergen (DP, AA, or PJ) had no significant effect on decane or nonadecane level (Figure S15A).

3.5 | Secondary Objectives

3.5.1 | Association Between Exhaled VOCs and Markers of Airway Inflammation

Five VOCs were selected for analysis: decane and nonadecane (from the predictive model) and acetone, styrene, and nonanal (from [19]). Subjects with FeNO ≥ 25 ppb at baseline displayed higher median levels of all five VOCs, with a significant association for decane ($p=0.005$), styrene ($p=0.012$), and nonanal ($p=0.005$) (Figure 3A). No significant correlations were found with FEV1, and no significant associations were observed with BEC or asthma symptoms (Figure S16A–C).

TABLE 1 | Baseline characteristics of the study population.

	Non-allergic subjects ($n=43$)		Allergic patients ($n=59$)			
	HC ($n=14$)	**NAR ($n=29$)**	**LAR ($n=31$)**	**AR ($n=28$)**	***p*-value[a]**	***p*-value[b]**
Age (years)[c]	33.4 ± 11.3	43.9 ± 16.8	39.6 ± 15.2	32.8 ± 11.0	0.181	**0.021**
Female, *n* (%)	10 (71.4%)	16 (55.2%)	24 (77.4%)	17 (60.7%)	0.385	0.155
BMI (Kg/m^2) [a]	24.0 ± 5.5	26.3 ± 5.0	24.9 ± 4.1	24.4 ± 4.4	0.428	0.183
Educational level:						
Incomplete secondary or less, *n* (%)	2 (14.3%)	8 (27.6%)	11 (35.5%)	6 (2.1%)		
Complete secondary, *n* (%)	1 (7.1%)	14 (48.3%)	10 (32.3%)	14 (50.0%)	0.430	0.674
University, *n* (%)	11 (78.6%)	6 (20.7%)	9 (29.0%)	7 (25.0%)		
Unknown, *n* (%)	0 (0%)	1 (3.4%)	1 (3.2%)	1 (3.6%)		
Current occupation:						
Managers-technicians, *n* (%)	11 (78.6%)	6 (20.7%)	7 (22.6%)	5 (17.9%)		
Skilled, *n* (%)	0 (0%)	5 (17.2%)	5 (16.1%)	4 (14.3%)		
Semiskilled-unskilled, *n* (%)	0 (0%)	5 (17.2%)	9 (29.9%)	8 (28.6%)	0.071	0.918
Unemployed, *n* (%)	3 (21.4%)	11 (37.9%)	8 (25.8%)	8 (28.6%)		
Unknown, *n* (%)	0 (0%)	2 (6.9%)	2 (6.5%)	3 (10.7%)		
Rhinitis duration (years)[c]	NA	10.3 ± 10.5	10.5 ± 8.9	11.8 ± 10.3	NA	0.720
Persistence of rhinitis:						
Intermittent, *n* (%)	0 (0%)	7 (24.1%)	6 (19.4%)	4 (14.3%)	NA	0.642
Persistent, *n* (%)	0 (0%)	22 (75.9%)	25 (80.6%)	24 (85.7%)		
Severity of rhinitis:						
Mild, *n* (%)	0 (0%)	6 (20.7%)	4 (12.9%)	6 (21.4%)		
Moderate, *n* (%)	0 (0%)	21 (72.4%)	24 (77.4%)	21 (75.0%)	NA	0.810
Severe, *n* (%)	0 (0%)	2 (6.9%)	3 (9.7%)	1 (3.6%)		
Asthma symptoms (yes), *n* (%)	0 (0%)	2 (6.9%)	4 (12.9%)	6 (21.4%)	0.068	0.276
FEV_1 (%) [a]	92.6 ± 16.4	98.2 ± 13.7	101.5 ± 15.2	100.8 ± 11.3	0.063	0.454
Blood eosinophil count (cells/μL)	147.9 ± 117.2	240.0 ± 221.3	187.1 ± 121.3	297.0 ± 210.4	0.071	0.092
FeNO (ppb) [a]	15.2 ± 9.9	20.4 ± 11.1	18.5 ± 12.5	42.1 ± 49.9	0.271	0.076
Total IgE (kU/L)[c]	48.5 ± 19.6	87.2 ± 150.1	80.0 ± 129.5	212.8 ± 241.0	0.059	**0.008**
sIgE (kU/L):						
D. pteronyssinus-sIgE	< 0.35	< 0.35	< 0.35	20.4 ± 29.2	NA	NA
Alternaria alternata-sIgE	< 0.35	< 0.35	< 0.35	3.49 ± 3.13	NA	NA
Parietaria judaica-sIgE	< 0.35	< 0.35	< 0.35	< 0.35	NA	NA
Positive diagnostic NAC-M:						
D. pteronyssinus (yes), *n* (%)	0 (0%)	0 (0%)	22 (71.0%)	24 (85.7%)	NA	NA
Alternaria alternata (yes), *n* (%)	0 (0%)	0 (0%)	7 (22.6%)	4 (14.3%)	NA	NA
Parietaria judaica (yes), *n* (%)	0 (0%)	0 (0%)	2 (6.5%)	0 (0%)	NA	NA

Note: Values are expressed as mean ± standard deviation (SD) or *n* (%), as appropriate. Bold digits denote comparison with statistical significance ($p < 0.05$).
[a]Comparison between allergic and non-allergic subjects.
[b]Comparison among rhinitis phenotypes; AR: allergic rhinitis; BMI: body mass index; *D. pteronyssinus*: *Dermatophagoides pteronyssinus*. FeNO: fractional exhaled nitric oxide; FEV_1: Forced Expiratory Volume in one second; HC: healthy control; LAR: local allergic rhinitis; NAC-M: nasal challenge with multiple allergens; NAR: Non-allergic rhinitis; sIgE: specific IgE.
[c]mean ± SD.

TABLE 2 | Environmental exposures and lifestyle habits across study groups.

	Non-allergic subjects($n=43$)		Allergic patients ($n=59$)			
	HC ($n=14$)	**NAR ($n=29$)**	**LAR ($n=31$)**	**AR ($n=28$)**	***p*-value[a]**	***p*-value[b]**
Smoking status:						
Current smoker, *n* (%)	0 (0.0%)	5 (17.2%)	5 (6.1%)	8 (28.6%)		
Previous smoker, *n* (%)	2 (14.3%)	6 (20.7%)	8 (25.8%)	3 (10.7%)	0.357	0.533
Never smoker, *n* (%)	12 (85.7%)	17 (58.6%)	17 (54.8%)	16 (57.1%)		
Frequency of alcohol consumption:						
Daily, *n* (%)	0 (0.0%)	0 (0.0%)	2 (6.5%)	2 (7.1%)		
≥Once a week, *n* (%)	7 (50.0%)	10 (34.5%)	7 (22.5%)	9 (32.1%)		
≥Once a month, *n* (%)	1 (7.1%)	7 (24.1%)	9 (29.0%)	7 (25.0%)	0.240	0.487
≥Once year, *n* (%)	2 (14.3%)	2 (6.9%)	0 (0.0%)	3 (10.7%)		
Never, *n* (%)	4 (28.6%)	9 (31.0%)	12 (38.7%)	6 (21.4%)		
Residence in a coastal city/ town (yes), *n* (%)	14 (100%)	24 (82.8%)	26 (83.9%)	26 (92.9%)	1.000	0.369
Proximity to high-traffic roads (yes), *n* (%)	10 (71.4%)	11 (37.9%)	14 (45.2%)	11 (39.3%)	0.684	0.833
Damp stains in the household (yes), *n* (%)	1 (7.1%)	3 (10.3%)	8 (25.8%)	10 (35.7%)	**0.013**	0.073
History of water leaks at home (yes), *n* (%)	0 (0.0%)	1 (3.4%)	6 (19.4%)	0 (0.0%)	0.233	**0.013**
Frequency of home ventilation:						
Daily, *n* (%)	14 (100%)	27 (93.1%)	30 (96.8%)	25 (89.3%)		
≥3 times a week, *n* (%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (3.6%)	0.418	0.379
≥Once a week, *n* (%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (3.6%)		
<Once a week, *n* (%)	0 (0.0%)	1 (3.4%)	0 (0.0%)	0 (0.0%)		
Physical activity:						
Very active, *n* (%)	1 (7.1%)	2 (6.9%)	0 (0.0%)	2 (7.1%)		
Quite active, *n* (%)	5 (35.7%)	4 (13.7%)	6 (19.4%)	8 (28.6%)		
Moderate active, *n* (%)	6 (42.9%)	13 (44.8%)	14 (45.2%)	8 (28.6%)	0.622	0.500
Not very active, *n* (%)	2 (14.3%)	7 (24.1%)	9 (29.0%)	9 (32.1%)		
Sedentary, *n* (%)	0 (0.0%)	2 (6.9%)	1 (3.2%)	0 (0.0%)		
Frequency of fast food consumption:						
Daily, *n* (%)	0 (0.0%)	1 (3.4%)	0 (0.0%)	0 (0.0%)		
≥Once a week, *n* (%)	2 (14.3%)	6 (20.7%)	7 (22.5%)	13 (46.4%)	0.134	0.186
≥Once a month, *n* (%)	12 (85.7%)	12 (41.4%)	12 (38.7%)	10 (35.7%)		
<Once a month, *n* (%)	0 (0.0%)	9 (31.0%)	11 (35.5%)	4 (14.3%)		
Pets keeping habit (yes), *n* (%)	6 (42.9%)	13 (44.8%)	14 (45.2%)	12 (42.9%)	1.000	0.984

Note: Values are expressed as *n* (%). Bold digits denote comparison with statistical significance ($p<0.05$).

[a]comparison between allergic and non-allergic individuals.

[b]comparison among rhinitis phenotypes. AR: allergic rhinitis; HC: healthy control; LAR: local allergic rhinitis; NAR: non-allergic rhinitis.

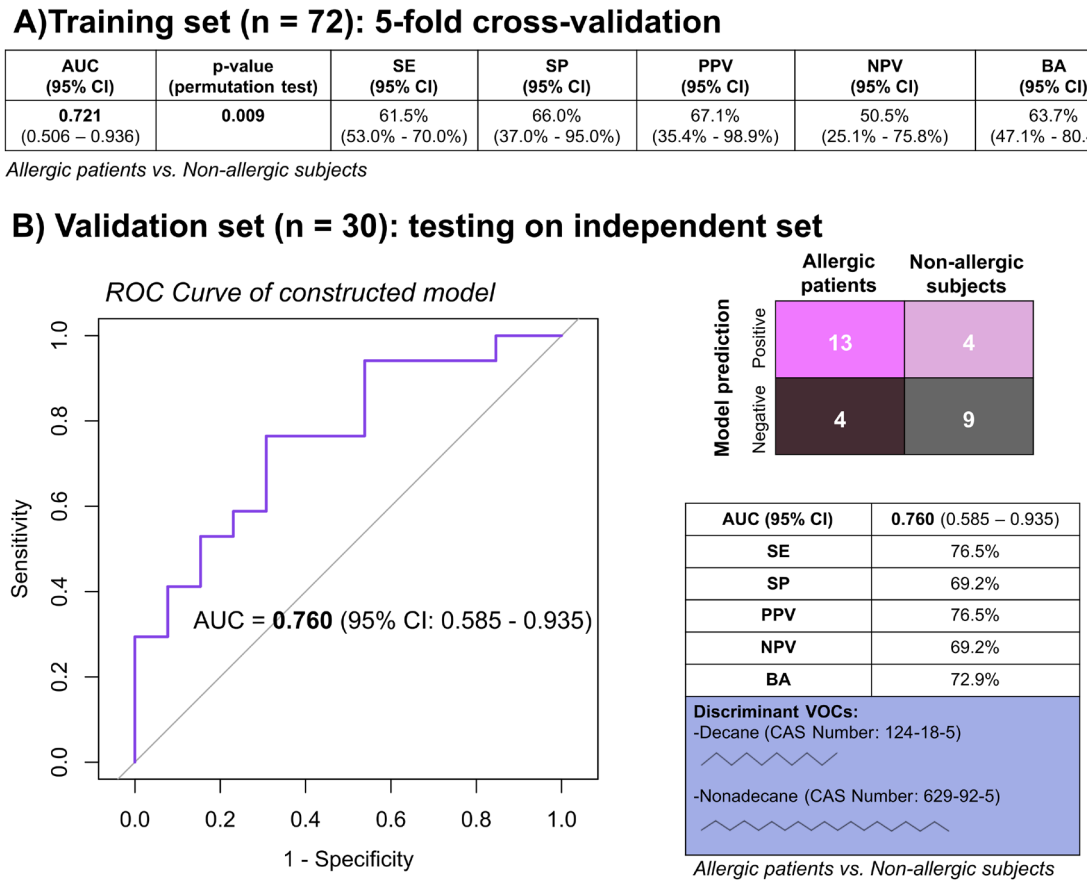

FIGURE 1 | Predictive model of respiratory allergic disease based on exhaled VOCs (Model I): Performance of the constructed model and selected VOCs. (A) 5-fold cross-validation on the training set and permutation test. (B) Testing on the validation set. AUC: Area under the curve; ROC: Receiver operating characteristic; 95% CI: 95% confidence intervals; SE: Sensitivity; SP: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; BA: Balanced accuracy; VOC: Volatile organic compound.

3.5.2 | Impact of the Nasal Allergen Challenge on the Exhaled Volatilome of Rhinitis Patients

We assessed the effect of NAC-S on the relative abundance of the five selected VOCs and FeNO levels. In allergic individuals, exhaled nonadecane showed a significant reduction 24 h after the provocation ($p = 0.012$), whereas no changes were observed in non-allergic subjects (Figure 4). FeNO levels also remained unchanged after the challenge. When stratifying allergic individuals by sensitizing allergen, a significant reduction in exhaled nonadecane 24 h post-NAC was observed in DP-allergic individuals ($p = 0.028$), but not in AA-allergic patients (Figure S17). The relative abundance of the other four VOCs and FeNO remained stable after the challenge in both allergen-specific subgroups.

No significant between-group differences in the predictive VOCs (decane and nonadecane) were detected 24 h after NAC-S (Figure 2B). Moreover, the presence of asthma symptoms, household dampness, water leaks, or the type of sensitizing allergen did not influence post-challenge levels of either compound (Figure S12B,S,1,3B and Figure S14B,S,1,5B). The presence of elevated FeNO (≥ 25 ppb) after allergen exposure did not influence the post-challenge levels of the five selected VOCs (Figure 3B). Notably, clinical responses to allergen exposure were fully concordant (100%) between NAC-M and NAC-S protocols across all participants.

4 | Discussion

This study aimed to explore the potential diagnostic utility of exhaled VOCs in chronic rhinitis. We identified a VOC signature, characterized by elevated levels of decane and nonadecane, with the potential to discriminate allergic (AR + LAR) from non-allergic (NAR + HC) individuals. Moreover, the same two VOCs showed a discriminatory signal between LAR and NAR individuals, with the former patients displaying significantly higher nonadecane than the latter individuals. As a secondary aim, we found that baseline levels of decane, styrene, and nonanal were significantly associated with FeNO ≥ 25 ppb, paving the way for their potential use as non-invasive biomarkers for T2 inflammation in future studies. Although nonadecane levels changed with the provocation in allergic patients only, NAC did not improve the discriminatory ability of VOCs for allergic or T2 inflammation.

The success of AIT largely relies on the accurate identification of allergic individuals, beyond the results of atopy tests [8–11]. When the clinical history is inconclusive, NAC can clarify the allergic etiology of rhinitis in atopic patients. The NAC is also the gold standard for LAR diagnosis [2]. Despite its well-established safety, reproducibility [31], standardized methodology [20], and validated cut-offs [32], NAC has practical limitations: it is a time-consuming procedure, requires specialized technical resources and extended medication wash-out periods [20], may

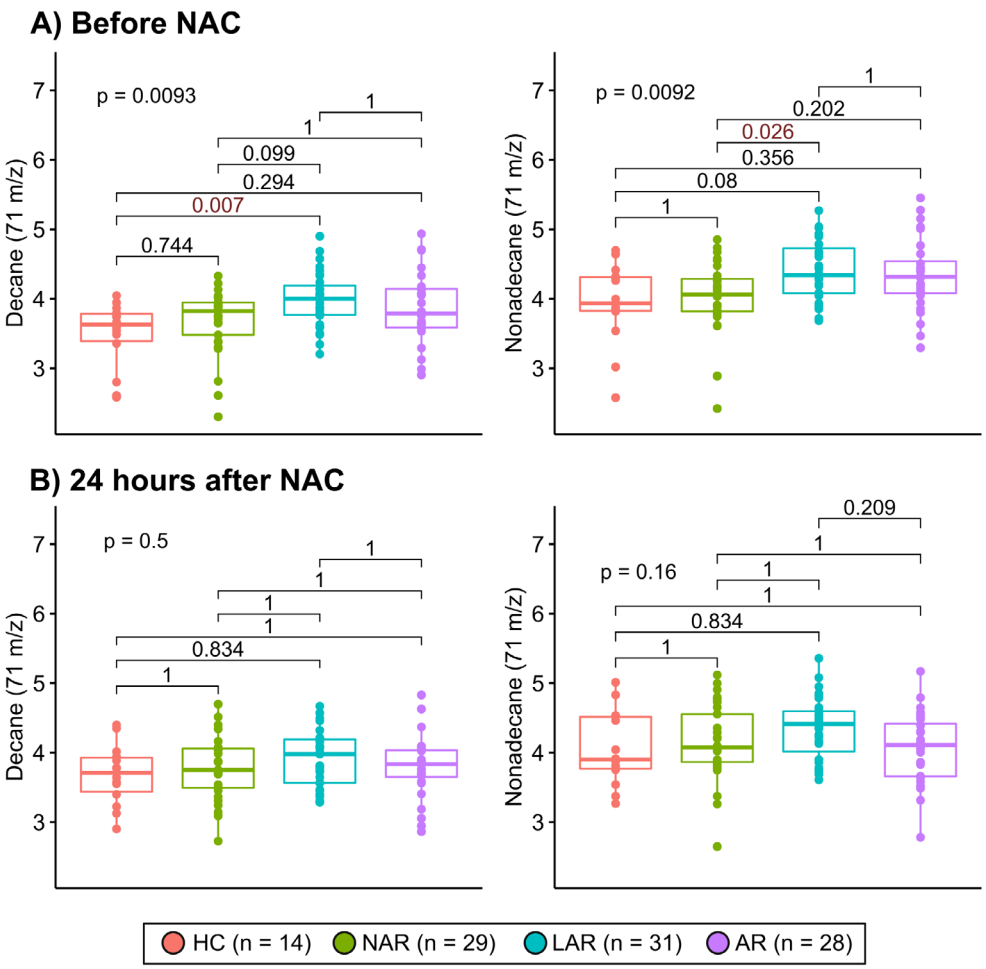

FIGURE 2 | Exhaled levels of the predictive VOCs across study groups. Boxplots show the relative abundance of decane and nonadecane in HC and patients with NAR, LAR, or AR. (A) Before NAC. (B) At 24 h post-NAC. AR: Allergic rhinitis; HC: Healthy control; LAR: Local allergic rhinitis; NAC: Nasal allergen challenge; NAR: Non-allergic rhinitis; VOC: Volatile organic compound.

induce troublesome symptoms, and is not reimbursed by some healthcare systems [12]. Alternative tools such as the basophil activation test (BAT) have shown promise as in vitro surrogates for NAC [33]. Nevertheless, the need for a fresh blood sample and the existence of non-responsive basophil phenotypes (10%–20% of individuals) represent practical and biological limitations [34]. Conversely, exhaled breath analysis is a non-invasive, age-independent, and patient-friendly technique, offering a new platform for biomarker identification.

Our exploratory results show that a combination of decane and nonadecane in exhaled air has the potential to identify allergic subjects. The predictive power of this model (Model I) is supported by a significant permutation test and consistent AUC values in both training and validation sets. This fact indicates a minimal risk of overfitting for Model I [13, 17, 35]. Consistent with previous works [26], Model I analyzed NAR and HC subjects together because both groups share the lack of IgE-mediated allergen-specific nasal reactivity, the key trait relevant to the primary study aim. This approach is appropriate for an exploratory analysis, given that normal reference values for most exhaled VOCs remain unknown. However, from a clinical perspective, the discrimination between LAR and NAR is more relevant as both phenotypes share the negativity of atopy tests [2]. In this context, Model IB suggests that exhaled decane and nonadecane might also differentiate LAR from NAR patients, although the absence of an independent validation split in this model represents a risk of overfitting. In any case, the allergy-specific VOC signature was particularly pronounced in LAR individuals, suggesting the utility of decane and nonadecane as non-invasive biomarkers for the selection of NAC and BAT candidates among non-atopic patients. Although decane was previously linked to asthma [17, 18], this is the first study to characterize a specific VOC signature in patients with NAC-confirmed airway allergy.

The clinical implementation of exhaled VOCs requires validation in larger cohorts including polyallergic patients and confirmed asthmatics, as well as the development of automated quantification devices. VOCs should not be used as a standalone diagnostic tool as they cannot identify the culprit allergen. Conversely, integrating VOCs with multi-omic approaches, established biomarkers, and clinical risk factors may help select suitable candidates for confirmatory tests such as NAC or BAT.

The higher baseline levels of exhaled decane (LAR vs. HC) and nonadecane (LAR vs. NAR) may reflect increased oxidative stress from mucosal inflammation. Chronic inflammation can trigger

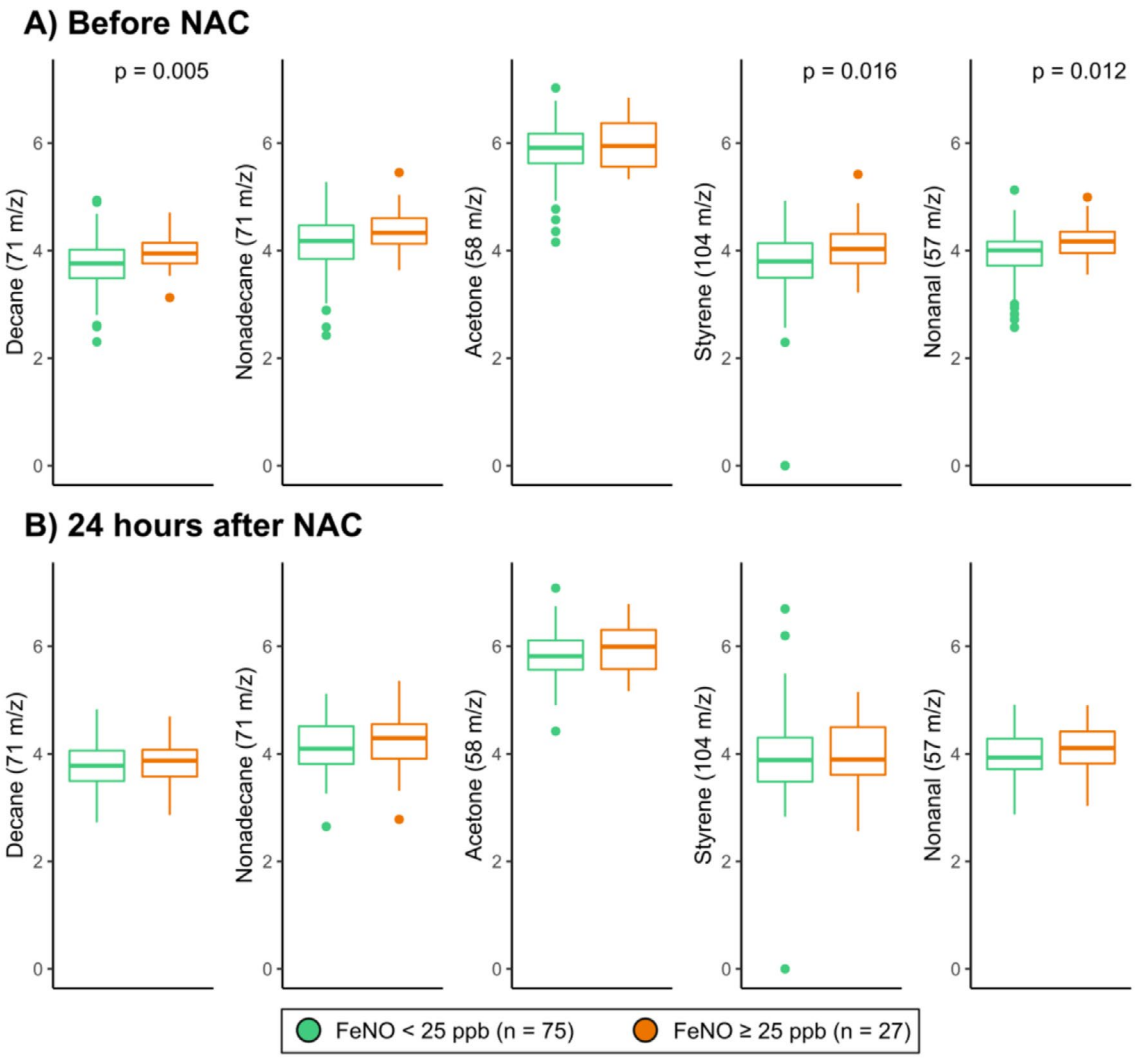

FIGURE 3 | Association between FeNO and exhaled VOCs. Subjects were categorized according to their FeNO value at baseline and 24 h post-NAC into < 25 and ≥ 25 ppb groups. Boxplots show the relative abundance of decane, nonadecane, acetone, styrene, and nonanal before (A) and 24 h after (B) NAC in both groups. FeNO: Fractional exhaled nitric oxide; NAC: Nasal allergen challenge; VOC: Volatile organic compound.

ROS production, which acts on cell membrane lipids (lipid peroxidation), generating by-products such as saturated hydrocarbons [36]. In any case, further mechanistic studies are required to elucidate the biological significance of exhaled VOCs [13].

Allergic patients usually display elevated BEC and FeNO, although these biomarkers do not discriminate between T2 allergic and T2 non-allergic inflammation [37]. Consistently, in our study, the FeNO-/BEC-based model (Model II) was outperformed by Models I and III in identifying allergic disease. The significant association of FeNO≥ 25 ppb with elevated baseline levels of styrene and nonanal is consistent with previous studies reporting a correlation between these VOCs, together with acetone, and sputum eosinophilia in asthmatics [19]. The absence of a significant association between acetone and T2 inflammation here may reflect its lower specificity, given that acetone is a ubiquitous VOC arising from multiple sources (e.g., ketone body metabolism, isopropanol oxidation, and intestinal microbiota) and influenced by factors such as diet and lifestyle [38]. In any case, our findings reinforce the potential of nonanal and styrene as non-invasive markers of T2 inflammation, with possible applications beyond rhinitis (e.g., phenotyping of asthmatics for biologicals [37]).

To our knowledge, this is the first study exploring the impact of allergen exposure on the exhaled volatilome in chronic rhinitis. A significant reduction in nonadecane was observed 24 h after a positive NAC in allergic and DP-allergic individuals, whereas no change occurred in NAR and HC subjects after a negative NAC. These findings might reflect the ability of DP to down-regulate specific metabolic pathways in allergic patients. The different behavior of DP- and AA-allergic patients may relate to the distinct inflammatory mechanisms triggered by each allergen (proteolytic activity for DP vs. IL-33 release for AA) or to the smaller sample size in the AA group [37]. In any case, a previous NAC did not improve the discriminatory capacity of exhaled VOCs, further suggesting their utility as baseline screening biomarkers for airway allergy.

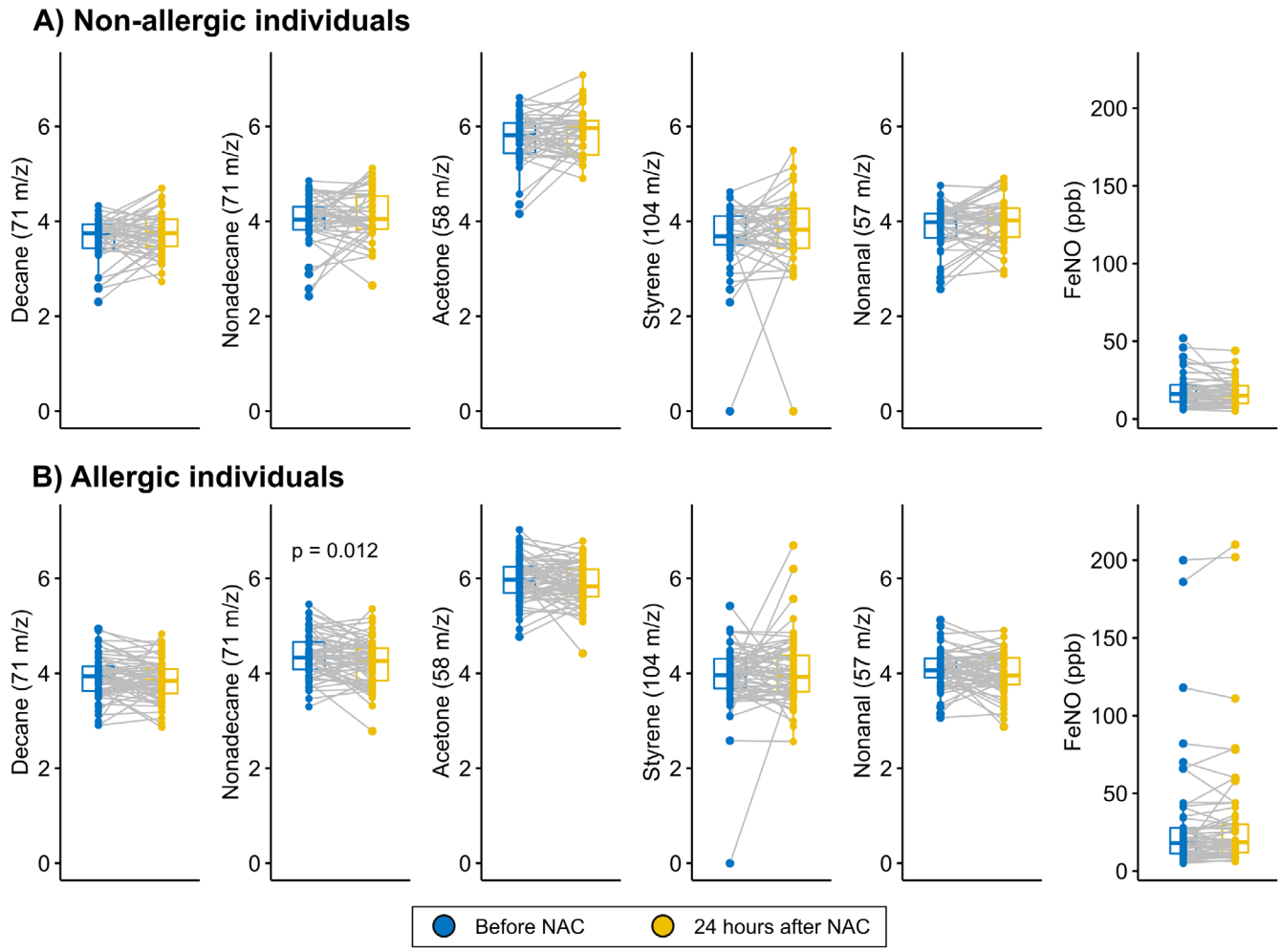

FIGURE 4 | Effect of the NAC on selected exhaled VOCs and FeNO. Paired box-and-scatter plots show the levels of decane, nonadecane, acetone, styrene, nonanal, and the FeNO value before and 24 h after NAC. (A) In non-allergic individuals (NAR and HC). (B) In allergic patients (AR and LAR). AR: Allergic rhinitis; FeNO: Fractional exhaled nitric oxide; HC: Healthy control; LAR: Local allergic rhinitis; NAC: Nasal allergen challenge; NAR: Non-allergic rhinitis; VOC: Volatile organic compound.

Besides its exploratory nature, this study has several limitations. No formal sample size was calculated, the overall study population is limited, and the validation set includes a relatively small number of subjects. However, the number of participants is comparable to most published exploratory breathomics studies in respiratory diseases [30]. Similarly, the proximity to the 0.50 limit of the lower bounds of the CI for the AUCs in Models I and IB warrants cautious interpretation of the results. Some environmental exposures were unevenly distributed among groups, but they had no measurable impact on the VOC profile. Moreover, although ambient air from the sampling room was analyzed, outdoor air pollution in the patients' environment was not quantified [39]. We focused on monosensitized patients with perennial rhinitis, as their clinical management is particularly challenging. We also assessed the nasal reactivity to prevalent perennial allergens, DP and AA, which are difficult to avoid in real-life settings. Nevertheless, validation of our findings will require studies with larger sample size and broader inclusion criteria. Allergen-induced changes in VOCs were only examined 24 h post-NAC. Thus, we cannot exclude earlier and transient changes in other VOCs. In this regard, future studies exploring the temporal kinetics of exhaled volatilome following NAC would provide valuable insights. Finally, as asthma was not formally diagnosed [21], conclusions regarding its influence on the VOC profile remain limited.

Despite their exploratory nature, our findings may inform future studies on the role of exhaled VOC analysis in refining diagnostic algorithms for airway allergy, particularly when clinical history and atopy tests are discordant. By guiding the selection of candidates for NAC or BAT, this approach might promote more efficient resource utilization while ensuring the timely identification of AIT candidates. Further studies in broader populations, including polysensitized individuals with confirmed asthma, are warranted to validate and refine the diagnostic utility of exhaled VOCs in routine practice.

Author Contributions

I.E.-G., C.R., T.D.P., and A.T.-M. designed the study and coordinated the work of the other authors. L.Z.-F., M.F., M.J.T., I.E.-G., C.R., and A.T.-M. recruited the participants, L.Z.-F. and A.T.-M. conducted the clinical procedures, and L.Z.-F., A.C.-A., C.J.A., and A.T.-M. collected

the biological samples. R.A.S.-M. and T.D.P. analyzed the breath samples and conducted the bioinformatic analysis. R.A.S.-M., I.E.-G., C.R., T.D.P., and A.T.-M. wrote the manuscript, which was finally reviewed and approved by the other authors.

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Conflicts of Interest

I.E.-G. has received honoraria for lectures and advisory activities from HAL Allergy, Allergy Therapeutics, Inmunotek, LetiPharma, Diater, ALK, Allergopharma, Chiesi, Gebro, GSK, AstraZeneca, Sanofi, Novartis, Abbvie, Celltrion and Viatrix. C.R. has received honoraria for lectures from Gebro, GSK, AstraZeneca, Immunotek, and Roxal. A.T.-M. has received honoraria from lectures fees from LetiPharma, ALK, GSK and AstraZeneca. The other authors declare no conflicts of interest in relation to this work.

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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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** Supplementary Information. **Figure S1:** Supplementary Figure 1. **Figure S2:** Supplementary Figure 2. **Figure S3:** Supplementary Figure 3. **Figure S4:** Supplementary Figure 4. **Figure S5:** Supplementary Figure 5. **Figure S6:** Supplementary Figure 6. **Figure S7:** Supplementary Figure 7. **Figure S8:** Supplementary Figure 8. **Figure S9:** Supplementary Figure 9. **Figure S10:** Supplementary Figure 10. **Figure S11:** Supplementary Figure 11. **Figure S12:** Supplementary Figure 12. **Figure S13:** Supplementary Figure 13. **Figure S14:** Supplementary Figure 14. **Figure S15:** Supplementary Figure 15. **Figure S16:** Supplementary Figure 16. **Figure S17:** Supplementary Figure 17.