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ORIGINAL ARTICLE

Asthma and Rhinitis

Small airway involvement in the late allergic response in asthma

H. Stenberg[1] | Z. Diamant[1,2] | J. Ankerst[1] | L. Bjermer[1] | E. Tufvesson[1]

[1]Faculty of Medicine, Department of Clinical Sciences Lund, Respiratory Medicine and Allergology, Lund University, Lund, Sweden

[2]Department of Clinical Pharmacy and Pharmacology, QPS-NL, The University Medical Center, University of Groningen, Groningen, the Netherlands

Correspondence
Henning Stenberg, Respiratory Medicine and Allergology, Department of Clinical Sciences, Lund University, Lund, Sweden.
Email: Henning.Stenberg@med.lu.se

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Summary

Background: Allergy and asthma are closely linked. Inhalation of allergen induces an early allergic response (EAR) within the airways of allergic asthmatic subjects, which is followed by a late allergic response (LAR) in approximately 50% of the subjects. The LAR is defined as a drop in forced expiratory volume in 1 second (FEV_1) from baseline usually occurring 4-8 hours after exposure and is believed to affect small airways. However, FEV_1 is insensitive to changes in small airway physiology.

Objective: Our aim was to investigate and compare the pathophysiological processes in large and small airways during the EAR and the LAR and to characterize subjects with both an EAR and a LAR (dual responders) versus those with an EAR only (single responders).

Methods: Thirty-four subjects with allergic asthma underwent an inhaled allergen challenge. Lung physiology was assessed by spirometry, impulse oscillometry (IOS), body plethysmography, inert gas washout, single breath methane dilution carbon monoxide diffusion and exhaled breath temperature (EBT), at baseline and repeatedly for 23 hours post-allergen challenge.

Results: Peripheral airway resistance, air trapping and ventilation heterogeneity were significantly increased in dual responders (n = 15) compared to single responders (n = 19) 6-8 hours post-challenge. Parameters of peripheral airway resistance and ventilation heterogeneity, measured with IOS and inert gas washout, respectively, correlated at baseline and during the allergic airway response in all subjects.

Conclusion: The LAR involves increased resistance and ventilation defects within the peripheral airways. Alternative definitions of the LAR including small airways pathophysiology could be considered.

Clinical relevance: Small airway dysfunction during the LAR suggests that dual responders may have more extensive airway pathology and underscores the relevance of small airways assessment in asthma.

KEYWORDS
airway physiology, allergen inhalation challenge, asthma, late allergic response, small airways

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1 | INTRODUCTION

Asthma is a chronic inflammatory disease of the airways, presenting with features including variable airway obstruction and bronchial hyperresponsiveness. Asthma and allergy are closely linked, and allergy-driven asthma is considered the most common phenotype.[1]

The allergen challenge has been developed to study the immunopathophysiology underlying asthma exacerbations and, subsequently, has been applied in targeted drug development.[2] In this asthma model, inhalation of a relevant allergen can induce an early allergic response (EAR) in allergic asthmatic subjects, characterized by an increased airway resistance mainly caused by airway smooth muscle constriction. In approximately 50% of subjects, the EAR is followed by a late allergic response (LAR) within 4-8 hours post-allergen challenge.[3] Subjects with both an EAR and a LAR are called dual responders. During the LAR, there is a T-helper 2-cell response within the airways, characterized by the presence of inflammatory cells and mediators[4-6] for at least 24 hours post-challenge, and an allergen-induced bronchial hyperreactivity that can last for several weeks.[2] While the EAR is thought to affect mainly large airways, the LAR is believed to involve the small airways as well.[7] Correspondingly, small airways dysfunction has been linked to worsened asthma control with increases in symptoms, exacerbations, severity of exercise-induced bronchoconstriction and late-phase allergic responses.[8] Therefore, understanding small airway pathophysiology in asthma, including the effects of drug interventions and the assessment methods, is becoming more and more important. So far, only very few studies have extensively compared different pathophysiological parameters during the LAR.[9]

Standard lung function testing (FEV_1) is insensitive to changes within the small airways.[10] Although plethysmography can provide indices of small airway involvement (eg air trapping), resistance measured by plethysmography cannot differ between large and small airways. The importance of a better understanding of methods proposed to assess small airway dysfunction, such as impulse oscillometry (IOS)[11] and inert gas washout,[12] has been highlighted as a step towards phenotyping and individualized treatment of patients with small airway disease, such as asthma and COPD.[13,14]

Our aims with this study were as follows: (i) to non-invasively assess small airway dysfunction in subjects with allergic asthma during the LAR, comparing different measuring techniques, (ii) to characterize dual responders versus single responders and (iii) to investigate alternative definitions of the LAR.

2 | METHODS

2.1 | Subjects

Thirty-four subjects with a doctor's diagnosis of allergic asthma according to GINA (Global Initiative for Asthma) guidelines[15] were included (Table 1). Subjects were not allowed to use short-acting beta2-agonists for at least 8 hours and long-acting beta2-agonists for at least 48 hours prior to any study-related assessments. All subjects had been clinically stable for at least 3 months on a daily dose of ≤400 μg budesonide (n = 16) or without inhaled corticosteroids (ICS) (n = 18). Subjects were instructed not to change their regular ICS use before participation and were allowed to use ICS in the mornings of the study days. None of the subjects were treated with anti-IgE, allergen-specific immunotherapy, oral corticosteroids, phosphodiesterase inhibitors, muscarinic or leukotriene receptor antagonists for at least 6 months pre-screening. Oral antihistamines were not allowed for at least 5 days prior to any visit. All subjects had a history of lower airway symptoms following exposure to the allergen used for the challenge and were otherwise in good general health. None were current or previous smokers. All subjects signed a written informed consent, and the Regional Ethics Review Board in Lund, Sweden, approved the study (2012/800).

TABLE 1 Subject characteristics

	Single responders (n = 19)	Dual responders (n = 15)
Sex (F/M)	9/10	8/7
Age (y)	27 (27-41)*	24 (22-31)*
Duration of asthma (y)	20 (10-24)	15 (13-21)
ACT (score)	22 (20-24)	22 (21-25)
Methacholine PD20 (μg)	208.1 (104.8-549.9)	228.0 (174.7-917.0)
Mannitol challenge, Pos/Neg (n)	9/7	6/8
Mannitol PD15 (mg)	288 (145-369)	401 (327-422)
Regular use of ICS (n)	6	10
Total IgE (kU/l)	126.0 (58.2-430.0)	86.8 (52.2-127.5)
Number of sensitizations (n)	5 (4-6)	4 (3-6)
Allergen used in challenge (n) (Cat/Horse/HDM/Birch/Grass)	10/4/2/2/1	8/2/1/2/2
Specific IgE for allergen used in challenge (kU/l)	3.8 (2.6-10.1)	6.6 (2.0-26.2)
SPT wheal diameter for allergen used in challenge (mm)	8 (7-11)	9 (8-11)
Allergen dose given (SQ-U)	250.3 (118.7-626.3)	386.6 (250.3-1303.1)

ACT, asthma control test; PD_{20}, provocative dose required to decrease forced expiratory volume in 1 s (FEV_1) by 20%; PD_{15}, provocative dose required to decrease FEV_1 by 15%; ICS, inhaled corticosteroids; HDM, house dust mite; SPT, skin prick test; SQ-U, standardized quality units.
Data presented as median (IQR), where applicable.
*$P < .05$: significant difference between groups.

2.2 | Study design

This was a single-centre, non-interventional, observational study, which consisted of three study days, separated by washout periods

of at least 72 hours (Figure 1). No more than 12 weeks passed between the first and the last visit. The study was performed between February 2013 and March 2016. Each subject's eligibility was assessed on a screening day. Subjects completed an asthma control test (ACT) questionnaire, consisting of five questions with answers scored from one to five; higher total scores meaning better asthma control.[16] Sensitizations to 10 allergens, including house dust mite (D pteronyssinus and D farinae), cat, horse, dog, alternaria alternata, cladosporium herbarum, grass, birch and ragweed pollen, were tested for by a skin prick test (ALK-Abello, Hørsholm, Denmark) and by serum IgE levels (RAST). All subjects had a positive skin prick test (wheal diameter ≥3 mm).[17] To characterize the direct bronchial hyperresponsiveness, all subjects performed a methacholine challenge test and those with a PD_{20} of >2000 μg were excluded from further testing. To further investigate the baseline inflammatory status using an indirect bronchial challenge, a mannitol inhalation test was performed on the second study day. Four subjects failed to perform the mannitol inhalation due to scheduling reasons. All visits were made outside the relevant pollen season for subjects sensitized to pollen. Subjects had to postpone visits if they had suffered from any respiratory infection during the last 3 weeks.

At the third study day, physiology assessments were performed at baseline and at predetermined time-points after the inhaled allergen challenge. When measurements coincided, they were performed in the order listed below to avoid interference. Baseline measurements and the inhaled allergen challenge were conducted at the same time of the day ±1 hour in all subjects.

2.2.1 | Methacholine inhalation challenge

A tidal-volume-triggered device (Aerosol Provocation System, APS, Erich Jaeger GmbH) was used for the methacholine challenge. Spirometry was first performed in triplicate with the best value being chosen as baseline. An inhalation of 9 mg/mL NaCl was done as a negative control prior to the challenge, and if FEV_1 measured after 2 minutes dropped ≥5% compared to baseline, the subject was excluded from further testing. Five inhalations with increasing doses (50, 150, 300, 600 and 900 μg, maximal cumulative dose 2000 μg) of methacholine were then administered, with spirometry being performed 2 minutes after each inhalation. The test was completed when FEV_1 dropped ≥20% compared to baseline.

2.2.2 | Mannitol inhalation challenge

Spirometry was performed in triplicate before the challenge. The best value was chosen as baseline. A mannitol powder kit (Aridol™; Pharmaxis, Frenchs Forest, NSW, Australia) was then used to deliver a maximal cumulative dose of 635 mg of mannitol, with eight incremental steps according to the manufacturer's instructions. FEV_1 was measured 1 minute after each inhalation. The challenge was completed and considered positive if/when FEV_1 dropped ≥15% compared to baseline.

2.2.3 | Exhaled breath temperature

Exhaled breath temperature (EBT), a suggested marker of airway inflammation, was measured with an X-halo (Delmedica Investments, Singapore)[18] before and at 0.5, 4, 7 and 23 hours post-allergen challenge. Measurements took 2-6 minutes to complete.

2.2.4 | Impulse Oscillometry system

Airway resistance and reactance were measured using a Jaeger MasterScreen Impulse Oscillometry System (IOS) (Erich Jaeger GmbH, Würzburg, Germany). Subjects wore a nose clip and pressed their palms against the cheeks to avoid upper airway shunting. Oscillometric pressure impulses with a frequency spectrum between 5 and 35 Hz were then superimposed on the subject's tidal breathing for at least 30 seconds. Mean values of resistance at 5 Hz (R5) and 20 Hz (R20), frequency dependence of resistance (R5-R20),

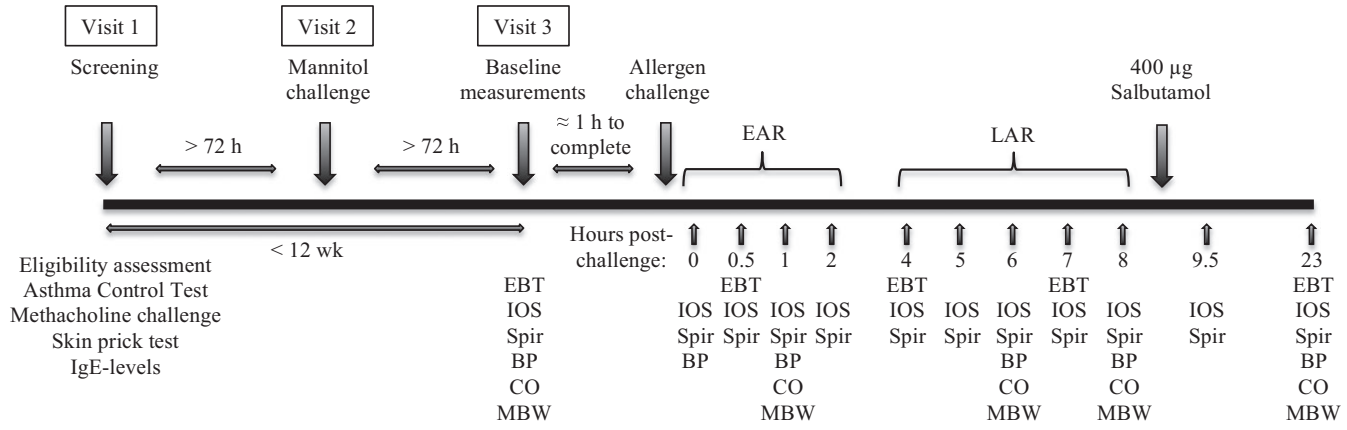


FIGURE 1 Study design. Assessments performed at each time-point are indicated below the line. EBT, exhaled breath temperature; IOS, impulse oscillometry; Spir, spirometry; BP, body plethysmography; CO, single breath CH_4 dilution carbon monoxide diffusion; MBW, multiple breath washout

reactance at 5 Hz (X5) and resonant frequency (F_{res}) were assessed. IOS was performed before the challenge and 0, 0.5, 1, 2, 4, 5, 6, 7, 8, 9.5 and 23 hours post-allergen challenge.

2.2.5 | Spirometry

Flow-volume spirometry was measured with a Jaeger MasterScope (Erich Jaeger GmbH, Würzburg, Germany) in accordance with European Respiratory Society (ERS)/American Thoracic Society (ATS) standards.[19] Percent of predicted (%p) values were calculated based on reference spirometric values of Crapo et al.[20] Spirometry was performed before the challenge and 0, 0.5, 1, 2, 4, 5, 6, 7, 8, 9.5 and 23 hours post-allergen challenge.

2.2.6 | Body plethysmography

Body plethysmography (MasterScreen Body, Erich Jaeger GmbH, Würzburg, Germany) measurements were performed according to ATS/ERS standards at baseline and 0, 1, 6, 8 and 23 hours post-allergen challenge.

2.2.7 | Carbon monoxide diffusion capacity

Single breath methane (CH_4) dilution carbon monoxide (CO) diffusion (MasterScreen PFT, Erich Jaeger GmbH, Würzburg, Germany) was measured at baseline and 1, 6, 8 and 23 hours post-allergen challenge. Predicted values of body plethysmography and diffusion capacity were calculated according to reference values of Quanjer et al.[21]

2.2.8 | Multiple breath washout

Multiple breath washout (MBW) of N_2 was measured with an Exhalyzer D (Eco Medics, Duernten, Switzerland) at baseline and 1, 6, 8 and 23 hours post-allergen challenge, as previously described.[22] The number of lung volume turnovers required to complete the test is termed lung clearance index (LCI), a measure of overall ventilation heterogeneity.[12] Analysis of the N_2 concentration during different phases of each breath provides more detailed information of where heterogeneity arises. S_{cond} and S_{acin} are indices of ventilation heterogeneity in the conductive and acinar airways, respectively. S_{cond} and S_{acin} were corrected for lung size and breathing pattern by multiplying values by tidal volume (V_T) for each breath (providing $S_{cond} \times V_T$ and $S_{acin} \times V_T$).

2.2.9 | Inhaled allergen challenge

For the inhaled allergen challenge, an automatic, inhalation-synchronized dosimeter jet-nebulizer (Spira Elektro 2, Respiratory Care Center, Hämeenlinna, Finland) was used. Subjects inhaled gradually increasing doses of a single diluted allergen extract (ALK-Abelló, Hørsholm, Denmark) by counted deep breaths. The choice of inhaled allergen (cat, horse, HDM, birch or grass pollen) was based on subject's history and skin prick test and/or serum IgE levels. Following baseline measurements, subjects inhaled gradually increasing doses of allergen, starting with 1.2 standardized quality units (SQ-U). FEV_1 was measured 5 and 10 min after completion of each inhalation step. If FEV_1 had not dropped ≥10% from baseline, the next inhalation (starting directly after the last spirometry) contained a fourfold increase in the dose of allergen. If FEV_1 dropped by 10%-15% from baseline, the next allergen dose was doubled, and if FEV_1 dropped 15-20%, FEV_1 was measured every 5 minutes for the following 30 minutes. If FEV_1 remained stable at a 15%-20% decline from baseline, the previous allergen dose was repeated. Whenever FEV_1 dropped ≥20% compared to baseline, the challenge was considered completed (=time-point 0), and no further allergen was inhaled. If a drop in FEV_1 ≥20% from baseline was not reached after a maximal cumulative dose of 20 000 SQ-U, the subject was excluded.

The EAR and the LAR were defined as the airway response (measured by FEV_1 and expressed as % fall from baseline) occurring 0-2 hours and 4-8 hours post-allergen challenge, respectively. After completion of the measurements at 8 hours post-allergen challenge (ie at approximately 9 hours post-allergen challenge), subjects inhaled 400 μg of salbutamol. Measurements were then conducted again 30 min after inhalation of salbutamol (=9.5 hours post-allergen challenge) and 23 hours post-allergen challenge.

2.3 | Statistical analyses

SPSS Statistics for Macintosh version 23.0 (IBM Corp, Armonk, NY, USA) was used for statistical analysis. All outcomes were expressed as % change from baseline. Comparisons between single and dual responders were made using the Mann-Whitney test, and paired comparisons were made with the Wilcoxon matched-pairs signed-rank test. Area under the curve (AUC) was calculated in GraphPad Prism for Macintosh version 7.0 (GraphPad Software Inc., La Jolla, CA, USA), based on the ratio of post-challenge value to baseline value. During the EAR and the LAR, AUC values were used for measurements where three or more measurements were recorded (R5, R20, R5-R20, X5), and the mean value was chosen where only two measurements were recorded (RV/TLC, R_{tot}, LCI, S_{cond}, S_{acin}). Spearman's rank correlation coefficient was used for correlation analyses. A P value of <.05 (two-tailed) was considered statistically significant. Results are expressed as median (IQR), where applicable.

3 | RESULTS

3.1 | Subjects

Subjects were categorized as dual responders if they had an EAR and a LAR; the LAR was defined as a ≥12% drop in FEV_1 from baseline occurring at any time-point during 4-8 hours post-allergen challenge. Subjects with an EAR only were defined as single responders. Based on these preset criteria, 19 subjects were identified as single responders and 15 subjects as dual responders. Single and dual responders differed in age but not in any other variable, including

duration of asthma, ACT-score, methacholine PD_{20}, mannitol PD_{15}, total IgE levels, specific IgE levels, SPT wheal diameter or number of sensitizations (Table 1). There were no significant differences in any of the baseline physiology parameters between single and dual responders (Table 2). No differences in clinical characteristics, methacholine or mannitol reactivity, or physiology parameters were found at baseline or at any time-point post-allergen challenge between subjects prescribed maintenance ICS therapy and subjects without ICS treatment (data not shown).

3.1.1 | Spirometry

The drop in FEV_1 was significantly greater in dual responders at each time-point 1-8 hours post-allergen challenge (Figure 2A), and in FVC at 30 min and at each time-point 2-8 hours post-allergen challenge (Figure 2B). After inhalation of salbutamol, the drop in FEV_1 (but not in FVC) was still significantly greater in dual responders. At 23 hours post-allergen challenge, both FEV_1 and FVC had once again dropped significantly more compared to baseline in dual responders vs single responders.

3.1.2 | Body plethysmography

Both residual volume (RV) and RV divided by total lung capacity (RV/TLC) were increased after allergen challenge but returned to baseline values in single responders at 6 hours post-allergen challenge. Dual responders had increased levels of RV and RV/TLC at 6, 8 and 23 post-challenge, significantly greater than the single responders (Figure 2C,D). The change in TLC was not different between single and dual responders at any time-point.

TABLE 2 Baseline physiology

	Single responders (n = 19)	Dual responders (n = 15)
FEV1 (L)	3.68 (3.35-4.31)	3.71 (3.47-4.13)
FEV1%p (%)	96.5 (88.7-103.6)	94.8 (92.5-103.2)
FVC%p (%)	102.5 (96.7-112.3)	105.4 (98.5-113.2)
R5 (kPa/L/s)	0.32 (0.28-0.37)	0.31 (0.28-0.34)
R20 (kPa/L/s)	0.29 (0.25-0.33)	0.29 (0.27-0.32)
R5-R20 (kPa/L/s)	0.03 (0.01-0.05)	0.02 (0.00-0.03)
X5 (kPa/L/s)	−0.09 (−0.11 to (−0.08))	−0.10 (−0.13 to (−0.08))
Fres (Hz)	10.24 (8.90-11.59)	10.64 (8.61-12.90)
RV%p (%)	102.7 (88.6-113.0)	94.4 (89.3-98.4)
TLC%p (%)	103.9 (98.7-107.1)	104.1 (98.0-107.9)
RV/TLC	0.28 (0.23-0.32)	0.26 (0.22-0.27)
LCI	7.2 (6.9-8.4)	7.5 (7.3-7.6)
Scond*V_T (/L)	0.022 (0.008-0.034)	0.014 (0.010-0.026)
Sacin*V_T (/L)	0.086 (0.054-0.107)	0.081 (0.061-0.103)
EBT (°C)	34.2 (34.0-34.5)	33.9 (33.3-34.2)

FEV_1, forced expiratory volume in 1 s; %p, percent of predicted value; FVC, forced vital capacity; R5, resistance at 5 Hz; R20, resistance at 20 Hz; X5, reactance at 5 Hz; F_{res}, resonant frequency; RV, residual volume; TLC, total lung capacity; LCI, lung clearance index; S_{acin}, ventilation heterogeneity of intra-acinar airways; S_{cond}, ventilation heterogeneity of conductive airways; V_T, tidal volume; EBT, exhaled breath temperature. Data presented as median (IQR).

3.1.3 | Impulse Oscillometry system

R5 was elevated in all subjects directly after the allergen challenge, but displayed a significantly higher increase in dual responders compared to single responders during the LAR (Figure 3A). R20 was also elevated in all subjects directly after the allergen challenge but was, in contrast to R5, similar in both groups at all time-points except post-salbutamol (Figure 3B). The frequency dependence of resistance (R5-R20) was significantly more increased from baseline in single responders compared to dual responders at all time-points 2-8 and 23 hours post-challenge, while there were no differences 0-1 hours post-challenge or post-salbutamol (data not shown). X5 and F_{res} also displayed significantly higher increases in dual responders during the LAR (Figure 3C,D). Although salbutamol removed the differences in R5, X5 and F_{res} responses between groups, significantly greater increases in these parameters were seen in dual responders again at 23 hours post-challenge (Figure 3A,C,D).

3.1.4 | Multiple breath washout

The increases in LCI and $S_{cond} \times V_T$ seen after the allergen challenge were significantly higher in dual responders during the LAR (Figure 4A,B). $S_{acin} \times V_T$ was not statistically different between groups at any time-point (Figure 4C).

3.1.5 | Body plethysmographic airway resistance, CO diffusion capacity and EBT

Total airway resistance (R_{tot}) and its inspiratory (R_{in}) and expiratory (R_{ex}) components were increased in all subjects directly after the challenge but only in dual responders at 6 hours post-challenge with a significant difference between groups (Fig. S1). R_{tot} was also significantly more increased 23 hours post-challenge in dual responders. RV and functional residual capacity (FRC) measured with CH_4 dilution CO diffusion were not statistically different between groups at any time-point (Fig. S2A,B). Diffusion capacity was significantly more reduced in dual responders compared to single responders at 1 hour post-allergen challenge but not when corrected for alveolar volume (Fig. S2C,D). EBT was significantly increased at 7 hours post-allergen challenge (34.4 [34.1-34.6]°C) in comparison with baseline (34.2 [33.7-34.4]°C) in all subjects ($P = .031$), but the increase did not differ between single and dual responders (Fig. S3).

3.1.6 | Correlations between physiology parameters

To compare suggested small airway indices derived from IOS, body plethysmography and MBW, correlations were assessed at baseline, during the EAR, the LAR and at 23 post-challenge (Table 3). R5-R20

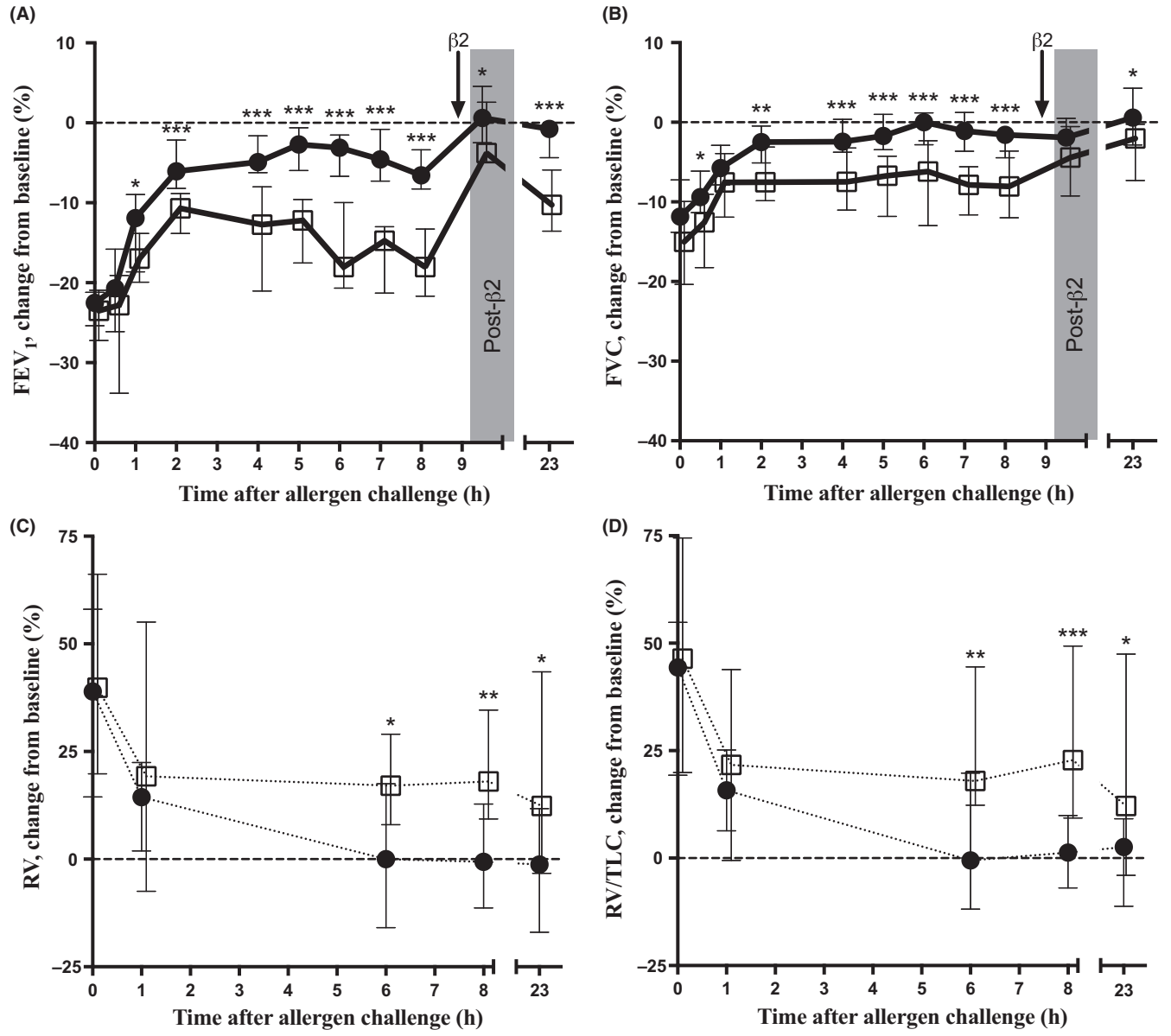


FIGURE 2 Spirometry and body plethysmography. FEV_1 (A), FVC (B), RV (C) and RV/TLC (D) in single and dual responders after the allergen challenge. β2, inhalation of 400 μg salbutamol, grey area marked with "Post-β2" indicates measurement performed during the effect of salbutamol. Data is presented as median (IQR). ●, single responders, □ , dual responders. Dotted lines are used where measurements are wider apart. *$P < .05$, ** $P < .01$, ***$P < .001$: significant difference between groups

correlated with S_{acin} at all time-points and with LCI at all time-points except at baseline. R5-R20 also correlated significantly with total plethysmographic airway resistance (R_{tot}) at all time-points. S_{cond} displayed several correlations with IOS parameters during the LAR and at 23 hours post-challenge but not at baseline or during the EAR. LCI correlated with all IOS parameters (except R20) as well as RV/TLC, R_{tot}, S_{cond} and S_{acin} during the LAR (Table 3).

3.1.7 | Alternative definitions of the LAR

To assess alternative definitions of the LAR involving the small airways, we investigated the subjects having either a drop in FEV_1 or an increase in small airway reactance or ventilation heterogeneity during the late-phase (change in $FEV_1 \geq 12\%$, in $X5 \geq 50\%$ or in $S_{cond} \geq 100\%$ at any time during the LAR. As there are no well-established cut-off values of abnormal X5 and S_{cond}, values were chosen that were comparable to results from previous reports on pathophysiological changes after airway provocations[23,24]). Twenty-two of 34 subjects fulfilled at least one of the conditions, with five subjects consistently above cut-off values of all three parameters. Seven of the 22 subjects would be classified as single responders based on FEV_1 criteria only but would qualify as dual responders if the LAR was defined by increased reactance (X5) or ventilation heterogeneity (S_{cond}) (Figure 5).

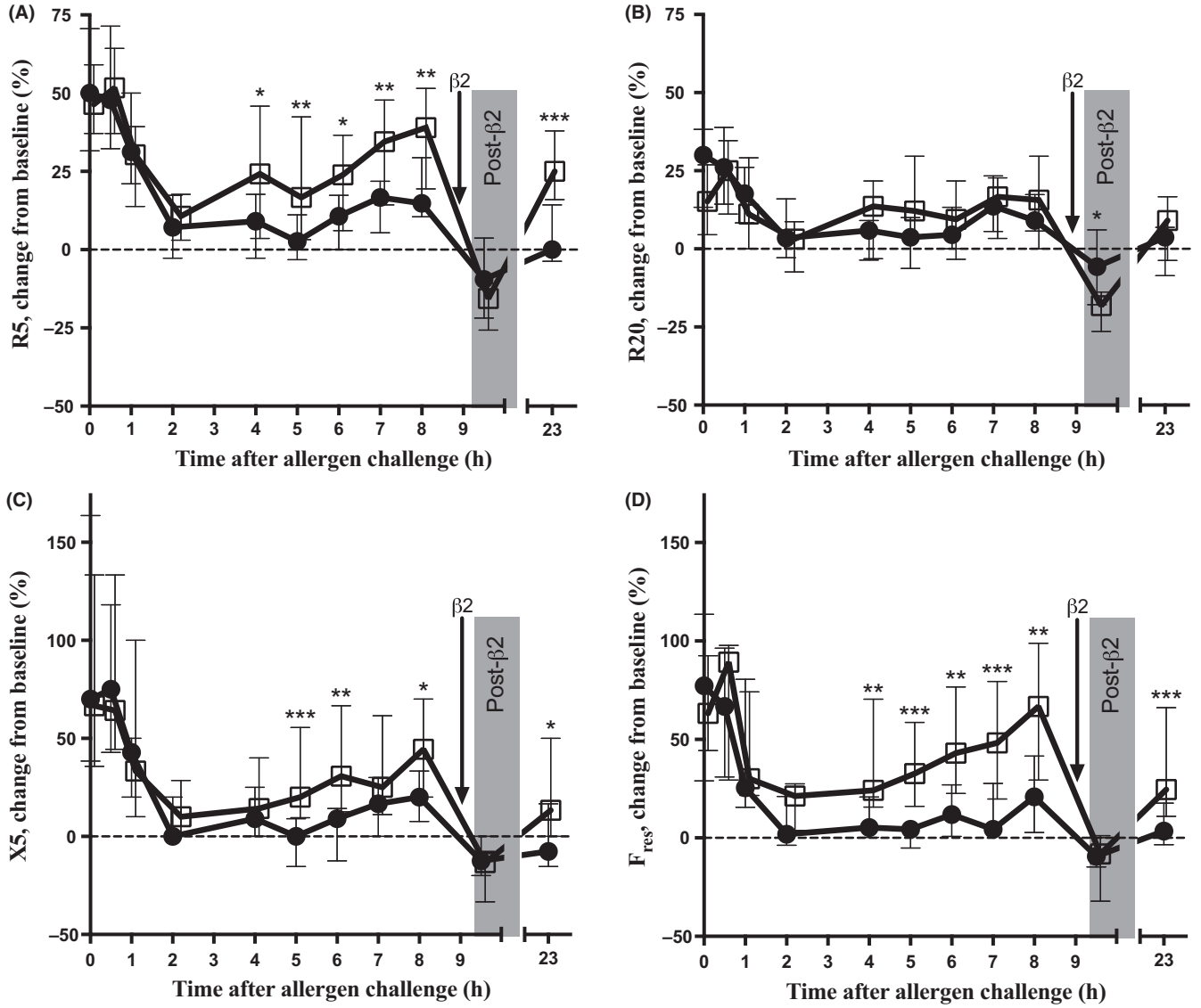
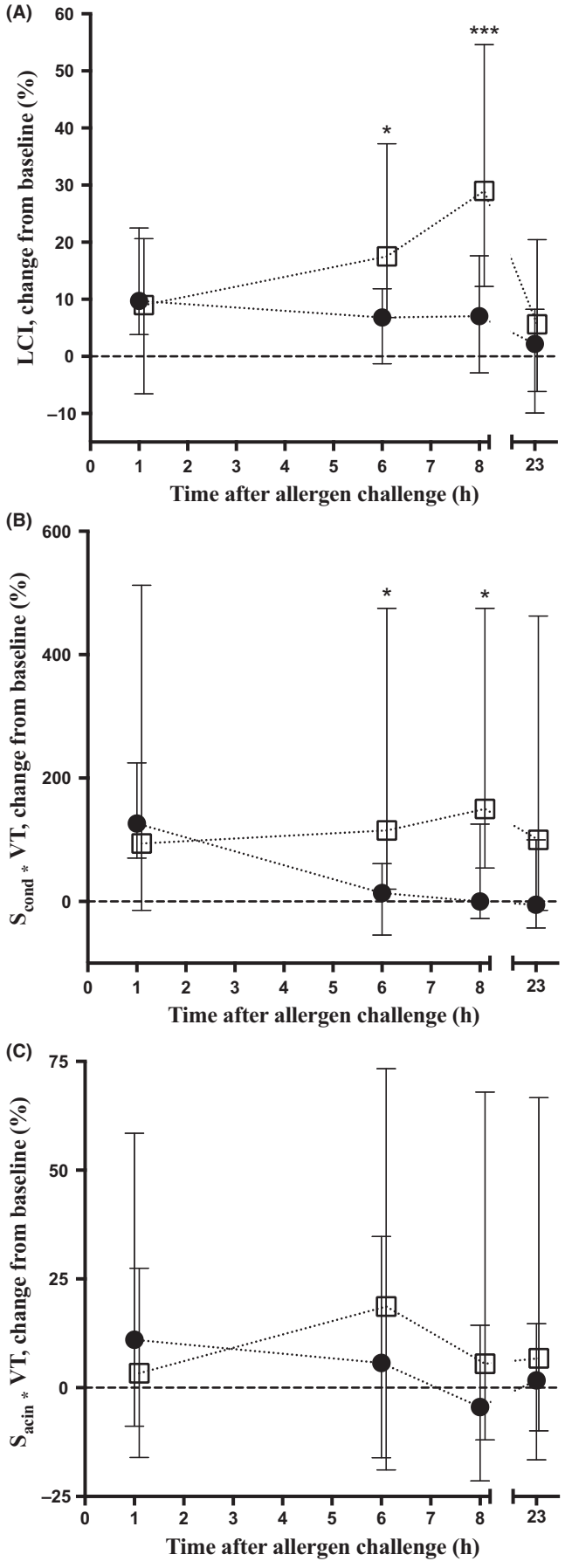


FIGURE 3 Impulse oscillometry. R5 (A), R20 (B), X5 (C) and F_{res} (D) in single and dual responders after the allergen challenge. β2, inhalation of 400 μg salbutamol, grey area marked with "Post-β2" indicates measurement performed during the effect of salbutamol. Data is presented as median (IQR). ●, single responders, □ , dual responders. *$P < .05$, **$P < .01$, ***$P < .001$: significant difference between groups

4 | DISCUSSION

In the present study, we found that in subjects who are traditionally considered as dual responders, resistance (R5) and reactance (X5, F_{res}) within the peripheral airways were increased during the LAR, while central resistance (R20) was not. In addition, air trapping and ventilation heterogeneity of the small conductive airways were increased during the LAR.

Our findings indicate that in dual responders mainly small airways are involved as opposed to single responders. So far, only scarce data exist on small airway involvement in the allergen-induced LAR.[9,25] Our data confirm previous findings and provide extension to the existing knowledge on the pathophysiology of the LAR. So far, there is no clear-cut formula to predict who will eventually develop a LAR. A large retrospective analysis of allergen challenges showed that dual responders are generally more reactive to methacholine at baseline and often show a larger drop in FEV_1 during the EAR, but results varied with the type of allergen used and dual responders in the group challenged with pollen actually needed higher cumulative doses of allergen than the single responders to develop an EAR.[26] Although related with more severe disease, the occurrence of a dual response cannot only be explained by disease severity, and our clinical data revealed no differences between the single and dual responders at baseline. We cannot, however, rule out the effect of maintenance therapy on baseline parameters in our study as the majority of the dual responders were on maintenance therapy with low-dosed ICS. Following allergen challenge, only incomplete bronchoprotection was achieved, while mainly high doses of ICS have been shown to fully protect against the LAR.[4] In addition, all subjects on maintenance ICS treatment were prescribed dry



powder inhalers, primarily targeting large airways.[27] Future studies should address whether ICS treatment targeting small airways is more effective in attenuating airway pathophysiology including small airway response during the LAR.

Administration of salbutamol reduced the differences between groups in FVC, airway resistance and reactance. To ensure adequate pulmonary deposition, salbutamol was administered using a pressurized metered dose inhaler in combination with a spacer.[28] Twenty-three hours post-allergen challenge the bronchodilator effect had worn off and the allergic response re-appeared, with significant differences between single and dual responders in FEV_1, FVC, R5, X5 and F_{res} (but not in R20), indicative of an inflammatory component in the dual responders. Further studies are required to determine the duration of the allergen-driven pathophysiological changes within the airways beyond 23 hours, and whether or how they differ in central versus peripheral airways.

DLCO/VA was unchanged after the inhaled allergen challenge and did not differ between groups. Our subjects are relatively young and completely healthy apart from a mild-to-moderate asthma. Greater disturbances in ventilation, exceeding those produced in our study, would probably be required to alter the diffusion capacity. EBT was significantly increased during the late-phase in all subjects but did not differ between groups. We have previously hypothesized that the increase in EBT seen after other types of airway challenges is a normal physiological reaction secondary to vasodilatation and increased blood flow.[29,30] It should be noted that the subjects had performed numerous repeated measurements of lung function during the study day, which might have affected EBT results.

The theoretical backgrounds for each of the methods suggested to detect peripheral airway dysfunction do not provide any detailed understanding on whether the different parameters reflect the same parts of the respiratory tract, or if an asthmatic small airway response affects the different outcomes in a similar way. Correlation analyses were made to increase this understanding, and several of the different parameters believed to indicate similar pathophysiological processes correlated with various degrees at baseline and during the different phases post-challenge. R5-R20, often termed frequency dependence of resistance, correlated significantly to R_{tot}, S_{acin} and LCI at most time-points before and after the inhaled allergen challenge and to S_{cond} 23 hours post-challenge. R5-R20 has in some publications been interpreted as an index of peripheral airway resistance,[31] while others have stated that it should be viewed as a heterogeneity of ventilation.[32] Based on our findings, we hypothesize that both peripheral airway resistance and ventilation heterogeneity are associated with an increased R5-R20.

FIGURE 4 Multiple breath washout. Lung clearance index (LCI) (A), ventilation heterogeneity in conducting airways ($S_{cond} \times V_T$) (B) and in acinar airways ($S_{acin} \times V_T$) (C) in single and dual responders. Data is presented as median (IQR). ●, single responders, □ , dual responders. *$P < .05$, ***$P < .001$: significant difference between groups

TABLE 3 Correlations between physiology parameters

Baseline	R5	R20	R5-R20	X5	RV/TLC	Rtot	LCI	Scond	Sacin
R5		**0.85*****	**0.61*****	**−0.47****	0.20	**0.55*****	−0.05	**0.36***	0.27
R20			0.18	**−0.48****	0.02	**0.40***	−0.08	0.22	0.14
R5-R20				−0.24	0.30	**0.34***	0.12	0.32	**0.41***
X5					**−0.35***	−0.31	−0.10	−0.09	−0.06
RV/TLC						0.25	−0.07	0.10	0.16
Rtot							0.32	0.21	**0.46****
LCI								0.01	0.30
Scond									0.05
EAR (0-2 h)	**R5 AUC**	**R20 AUC**	**R5-R20 AUC**	**X5 AUC**	**RV/TLC Mean**	**Rtot Mean**	**LCI 1 h**	**Scond 1 h**	**Sacin 1 h**
R5 AUC		**0.57*****	0.33	**0.49****	0.22	0.20	0.17	0.03	0.30
R20 AUC			−0.32	0.14	−0.10	−0.28	−0.12	−0.15	−0.22
R5-R20 AUC				**0.60*****	0.28	**0.54*****	**0.40***	0.27	**0.65*****
X5 AUC					0.16	0.27	0.21	**0.36***	**0.37***
RV/TLC Mean						0.19	**0.37***	0.12	0.25
Rtot Mean							0.12	0.03	**0.61*****
LCI 1 h								**0.62*****	0.19
Scond 1 h									0.02
LAR (4-8 h)	**R5 AUC**	**R20 AUC**	**R5-R20 AUC**	**X5 AUC**	**RV/TLC Mean**	**Rtot Mean**	**LCI Mean**	**Scond Mean**	**Sacin Mean**
R5 AUC		**0.76*****	0.12	**0.60*****	0.19	0.24	**0.42***	**0.38***	0.04
R20 AUC			−0.06	**0.40***	0.05	0.08	0.17	**0.35***	−0.26
R5-R20 AUC				0.33	0.08	**0.45****	**0.51****	0.29	**0.58*****
X5 AUC					0.13	**0.38***	**0.42***	**0.53****	0.29
RV/TLC Mean						0.14	**0.52****	0.28	0.13
Rtot Mean							**0.50****	0.29	**0.45****
LCI Mean								**0.46****	**0.37***
Scond Mean									−0.07
23 h	**R5**	**R20**	**R5-R20**	**X5**	**RV/TLC**	**R_{tot}**	**LCI**	**Scond**	**Sacin**
R5		**0.68*****	**0.63*****	**−0.70*****	0.31	**0.65*****	0.21	**0.49****	0.18
R20			0.05	**−0.45****	0.09	**0.38***	−0.08	0.24	0.04
R5-R20				**−0.51****	**0.35***	**0.42***	**0.35***	**0.43***	**0.40***
X5					−0.30	**−0.56*****	−0.23	**−0.53****	−0.16
RV/TLC						0.08	0.31	**0.45****	0.27
Rtot							**0.39***	**0.36***	0.23
LCI								0.28	0.29
Scond									0.05

R5, resistance at 5 Hz; R20, resistance at 20 Hz; X5, reactance at 5 Hz; RV, residual volume; TLC, total lung capacity; R_{tot}, plethysmographic total airway resistance; LCI, lung clearance index; S_{acin}, ventilation heterogeneity of intra-acinar airways; S_{cond}, ventilation heterogeneity of conductive airways. During the EAR and the LAR, AUC values were used for measurements where three or more measurements were recorded (R5, R20, R5-R20, X5), and the mean value was chosen where only two measurements were recorded (RV/TLC, R_{tot}, LCI, S_{cond}, S_{acin}). Note that absolute values of X5 are always negative in sign, while the AUC of X5 is positive in sign. AUCs are always considered positive in sign, even when going below the baseline. Spearman's rho is reported, and significant correlations are highlighted in grey.

*$P < .05$, **$P < .01$, ***$P < .001$: significant correlation.

Ventilation heterogeneity has been demonstrated in asthma and has been linked to increased airway hyperresponsiveness.[33] We found that S_{cond}, but not S_{acin}, was elevated during the LAR. Similar to these findings, Thompson *et al*[34] showed that S_{cond} was significantly higher in asthmatic patients with an exacerbation compared to patients with stable disease. They also showed that S_{acin} (but not S_{cond}) correlated inversely with FEV_1%p at baseline. This was also seen in the present study, indicating that intra-acinar airways are affected in stable asthmatic disease. Also, increased air trapping (RV/TLC) was observed during the LAR and was inversely correlated to FVC, similar to findings by Sorkness et al.[35] Correlations between RV/TLC and LCI were seen both during the EAR and the LAR. The

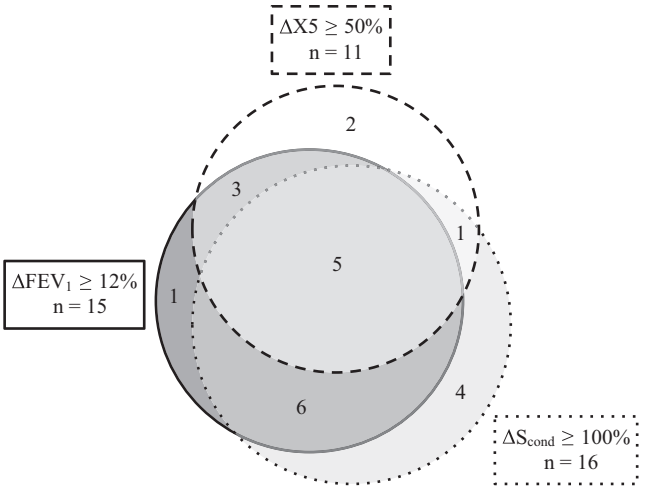


FIGURE 5 Alternative definitions of the LAR. Number of subjects categorized as dual responders based on three different definitions of the late allergic reaction: change in $FEV_1 \geq 12\%$ (dark grey), change in $S_{cond} \geq 100\%$ (grey) or change in $X5 \geq 50\%$ (white) at any time 4-8 h post-challenge, compared to baseline. The numbers of subjects that match one or several definitions are reported within the circles or within their overlapping fields, respectively

correlation between RV/TLC and S_{cond} at 23 hours post-challenge might indicate that ventilation heterogeneity and air trapping occurring in an allergic asthmatic exacerbation stem primarily from the conducting airways. Ventilation heterogeneity and airway closure are different degrees of a similar process, and even a minor heterogeneity of bronchoconstriction may affect adjacent airways and potentially induce a sequence of airway closure.[36] The fact that LCI correlated to all other parameters except R20 during the LAR indicates that ventilation heterogeneity is an important feature linked to several aspects of the pathophysiology of allergic asthmatic exacerbation.

Finally, as the LAR quite extensively affects the peripheral airways, one could question why its definition is based on changes in FEV_1 only. We investigated whether the same subjects would be defined as dual responders if alternative definitions, based on peripheral airway reactance or ventilation heterogeneity, were used. Although there was a substantial overlap, almost one third of the subjects would be categorized as single responders using only FEV_1, when they in fact displayed a dual response in reactance and/or ventilation heterogeneity. We therefore propose that IOS and MBW could be used to detect and define a LAR involving the small airways, but further studies will be needed to evaluate this concept. Previous studies have shown the clinical importance of small airway dysfunction in asthma. The fact that we have demonstrated the same dysfunction during the LAR, generally considered an accurate model of naturally occurring asthma, supports the notion that there is relevance in assessing the small airways.

In summary, our data indicate that during the allergen-induced LAR, small airway involvement prevails to central airway involvement in dual responders. From our data, it cannot be concluded whether the LAR is related to more severe disease as the majority of dual responders were on ICS maintenance therapy. Future studies need to address the effectiveness of targeted therapies on small airway dysfunction during the LAR.

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CONFLICTS OF INTEREST

Zuzana Diamant has received paid consultancy from Gilead, Aerocrine, HAL Allergy, Boehringer-Ingelheim and Acucort. The other authors report no conflict of interest.

AUTHOR CONTRIBUTION

HS acquired the data, performed all analyses and drafted the manuscript. ZD and ET assisted in drafting the manuscript. JA and LB assisted with inclusion and medical examination of subjects. All authors participated in creating the study design, revised the manuscript critically during drafting and have given final approval of the version submitted.

ORCID

H. Stenberg http://orcid.org/0000-0002-6959-3380

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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