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Nasal IL-13 production identifies patients with late phase allergic responses

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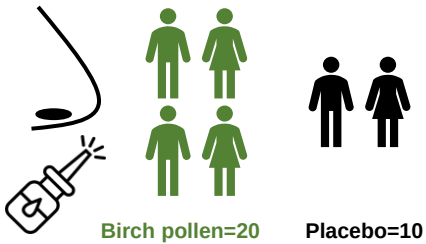
Study design



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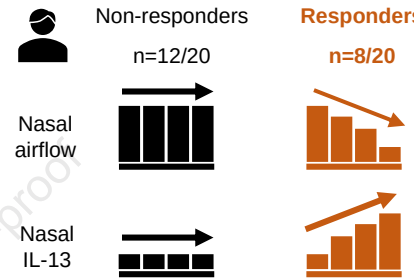
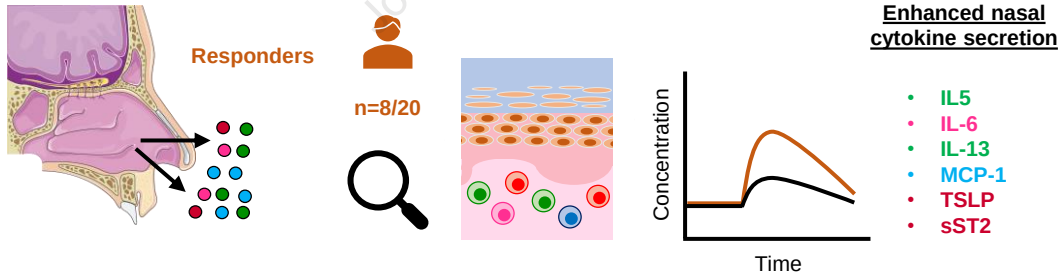


Late phase responders



IL: Interleukin, sST2: Secreted IL-33 receptor (IL1RL1), EDN: Eosinophil derived neurotoxin, MCP-1: Monocyte chemoattractant protein-1, TSLP: Thymic stromal lymphopoietin

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HK, ICS and ESC are employees of AstraZeneca and may own stock or stock options.

MM and EP are employees of Thermo Fisher Scientific and may own stock or stock options.

AUTHOR CONTRIBUTION STATEMENT

RV, VNL, JED, NJC, RR, RST and TTH designed the study. JED, VNL, RV and NJC wrote the study protocol and gained ethical approval for the study. JED and NJC recruited all the participants and JED, NJC, SVM, VS, JT, KG and SS carried out the study. NJC, JED, VS, SVM, RST, HK, ICS, ESC, EP, MM, MFT, RF, GR and TP performed the experiments. NJC, JED, SVM, RV, RST, RR and TTH performed the analysis. NJC, SVM, JED, MZ, RV and VNL wrote the manuscript. All authors critically revised the manuscript together.

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ABSTRACT

Background: There is limited knowledge on how local cytokine secretion patterns after nasal allergen challenge correlate with clinical symptoms especially with regards to the "late allergic response" (LAR) which occurs in approximately 40-50% of allergic patients.

Objective: In this study we aimed to characterise the immunological and clinical nasal responses to birch pollen allergen challenge with a special focus on the LAR.

Methods: In this randomised double-blinded placebo-control trial, birch pollen allergic participants were challenged with pollen extract (n=20) or placebo (n=10) on three consecutive days. On days one and three nasal secretions were collected at selected time points over a 24h time course for the measurement of 33 inflammatory mediators. Clinical responses were determined through subjective symptom scores and objective nasal airflow measurements.

Results: Provoked participants had significantly greater clinical responses and showed significant increases in tryptase and sST2 within minutes compared to placebo. Eight out of 20 provoked participants displayed high IL-13 levels 2-8 hours after allergen provocation. This group also showed significant changes in clinical parameters, with a secondary drop in nasal airflow measured by peak nasal inspiratory flow and increased symptoms of nasal obstruction which significantly differed from IL-13 non responders at 6 hours.

Conclusion: IL-13 response status correlates with cytokine and clinical responses in the late phase after allergen provocation.

Clinical implication: In the future, the analysis of IL-13 responses in allergic individuals upon allergen-challenge could be a promising biomarker for diagnosis of late phase responders.

Abstract Word Count: 241

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Capsule summary: The analysis of nasal IL-13 responses after allergen-challenge could be used as a biomarker predicting the development of late phase responses in the nose in allergic patients.

ClinicalTrials.gov ID: NCT03644680

Key words: Allergic Rhinitis, Birch Pollen Allergy, Allergen, Biomarker, Late Allergic Response, Nasal Allergen Challenge, Cytokine Responses

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ABBREVIATIONS

EAR: Early Allergic/Phase Response

LAR: Late Allergic/Phase Response

BPE: Birch Pollen Extract

NC: Nasal Challenge

AR: Allergic Rhinitis

IL-13^{R}: IL-13 Responder

IL-13^{NR}: IL-13 Non-Responder

IL: Interleukin

APRIL: A Proliferation-Inducing Ligand

BAFF: B Cell Activating Factor

IFN: Interferon

sST2: Serum stimulation-2

TNF: Tumour Necrosis Factor

TSLP: Thymic Stromal Lymphopoietin

EDN: Eosinophil-Derived Neurotoxin

MPO: Myeloperoxidase

NGAL (LCN2): Neutrophil Gelatinase-Associated Lipocalin (LCN2: Lipocalin 2)

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor

GRO: Growth-Regulated Oncogene

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MCP: Monocyte Chemotactic Protein

VAS: Visual Analogue Scale

TNSS: Total Nasal Symptom Score

PNIF: Peak Nasal Inspiratory Flow

BAT: Basophil Activation Test

Ig: Immunoglobulin

NaCl: Sodium Chloride

AUC: Area under the Curve

MLF: Mucosal Lining Fluid

SPT: Skin Prick Test

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1. INTRODUCTION

Allergic rhinitis is a major health problem that is increasing in global incidence, affecting around 30% of the world population. [1]

Clinical studies exploring the kinetics of the allergic response through nasal allergen challenges have highlighted the sequence of events occurring systemically and in the nasal mucosa. [2, 3, 4] Recent innovations in nasal mucosa sampling techniques [5] have considerably deepened our understanding of nasal immune responses.

In both allergic asthma and allergic rhinitis, the allergic response can show a biphasic kinetic in susceptible subjects as follows: The early allergic response (EAR) encompasses a very sharp rise in nasal symptoms within the first hour after allergen exposure. This occurs due to mast cell degranulation caused by allergen recognition by surface immunoglobin E (IgE). Mast cell mediators such as histamine, PGD2 and tryptase are detectable very rapidly, within the first 10 minutes after allergen exposure. [3, 6, 7, 8, 9] Additionally, the allergens themselves can compromise the epithelial barrier leading to the release of alarmins such as IL-33, IL-25 or TSLP. [6]

The second phase, termed the late allergic response (LAR) occurs two-eight hours (h) after allergen exposure in certain patients. [10] Approximately 50% of allergic rhinitis patients experience a symptomatic LAR after nasal allergen exposure. [11, 12, 13, 14, 15]

The nasal LAR is characterised mainly by an increase in nasal obstruction after initial recovery. [10] Events in the EAR lead to the release of vasoactive mediators which cause tissue oedema and the recruitment of a type two inflammatory infiltrate characterised by basophils, eosinophils, and T helper two (Th2) cells. [16, 17] These cells then produce and release type two cytokines such as IL-4, 5 and 13 as well as other pro-inflammatory mediators. [10] Patients responding with high levels of nasal IL-13 in the LAR after grass pollen challenge showed

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high IL-5 levels and upregulation of genes associated with type two inflammation as well as elevated baseline IL-33 levels. [3] Further characterisation of this subgroup of patients is important as different treatments have been shown to be effective for the EAR and LAR: While most commonly used antihistamines such as cetirizine - though very effective in combating the symptoms generated as a result of mast cell degranulation - have shown no effect on the symptoms in the LAR, glucocorticoids provide the most alleviation of symptoms in this phase. [18, 19] However, this is not the case for rupatadine, a second-generation antihistamine displaying anti-PAF as well as anti-H1R activity, which has been reported to ameliorate nasal congestion, the major symptom in LAR [20, 21, 22].With the advent of innovative but costly biological treatments, biomarkers to help define patients undergoing a LAR would greatly assist the success of these new forms of treatments.

Although controlled nasal allergen challenge is a well-established model, [2, 4] our knowledge on cytokine profiles after allergen challenge especially with regards to susceptibility to the LAR is limited. Here, we closely assessed clinical and nasal cytokine responses in 30 birch pollen allergic participants undergoing three consecutive nasal challenges. Nasal mucosal lining fluid (MLF) was collected during a 24 hour time course after nasal provocation with birch pollen extract (BPE) or placebo on days one and three. IL-13 responses after allergen challenge above levels seen in placebo challenged participants were identified as biomarker for identifying the development of an LAR which could be used for personalized medicine approaches in the future.

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2. METHODS

2.1. Study Design

For an overview of the study design please refer to Figure 1 and for a more detailed description please refer to the online supplements. For an overview of the recruited participant characteristics please refer to Table 1 and Table E1 in the Online Repository. Only participants who reported allergic rhinitis symptoms during the birch pollen season in Austria over at least two consecutive seasons, who also had a positive skin prick test and allergen-specific IgE to Bet v 1 were defined as having birch induced allergic rhinitis and were therefore included in this study. All participants reported the use of symptomatic medications during the birch season. Participants who showed discomfort or immediately sneezed after the application of nasosorptions during the screening visit were excluded from the study to avoid the inclusion of participants with nasal hypersensitivity.

2.2. Patient Randomisation and Controlled Nasal Allergen Exposure

This study was carried out at the University Department of Otorhinolaryngology at the General Hospital of Vienna, Austria. In this double-blind placebo-controlled nasal provocation study (**NCT03644680**), participants were randomised to either receive placebo or the BPE. They were stratified according to their Bet v 1 specific IgE levels and randomised in a 2:1 ratio (BPE; n=20, placebo; n=10).

The birch pollen season in Austria is between March-May with trees containing cross-reactive pollen allergens blooming a little earlier in January-February. Therefore, controlled nasal provocation was performed on three consecutive days well outside of the pollinating seasons (October) with the same dose of either placebo (100µl of vehicle control (0.9% NaCl) solution in each nostril per provocation or birch pollen extract (100µl of birch pollen extract (Allergopharma, Reinbeck Germany)) containing 20µg/ml of Bet v 1 in each nostril per

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provocation). Birch pollen extract was all ordered from the same LOT number (T7004903-DE) to ensure consistency between samples and Bet v 1 concentration was determined by sandwich ELISA using purified Bet v 1 as standard as previously described. [23] For delivery of challenge solutions Unidose Aptar nasal spray devices were used (AptarGroup Inc, Crystal Lake, Illinois, United States). On provocation days one and three participants underwent a 24-hour sampling time course (Figure 1A). During the provocation time course participants were strictly asked to refrain from taking symptomatic medications themselves.

2.3. Nasal Sampling

Nasosorptions (Nasosorption FX-I, Hunt Developments (UK) Limited, Midhurst, West Sussex, United Kingdom) were used for the collection of MLF and processed as previously described. [5]

2.4. Skin Prick Test

All participants were screened with a panel of 15 inhaled allergen extracts, including birch pollen using a commercial skin prick test set from Bencard Allergie (Munich, Germany). Tests were performed on the patient's forearms as previously described. [24, 25] For a list of the aeroallergens please see the online repository.

2.5. Cytokine Assays

Cytokine and granule protein concentrations in MLF and were measured using MSD multiplex U-Plex, R&D System's Quantikine ELISA, ProQuantum high-sensitivity immunoassays and ImmunoCAP, according to the manufacturer's protocol. All measurements were performed in triplicates. For a list of all the tested cytokines and further details please refer to the online repository.

2.6. Measurement of Clinical Parameters

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Patient's nasal air flow was assessed using an In-Check Nasal portable inspiratory flow (PNIF) meter (Clement Clarke International, Harlow, United Kingdom), a widely used tool for the objective assessment of nasal airflow in allergic rhinitis. [26]

For the assessment of nasal symptoms, the widely used modified Total Nasal Symptom Score (TNSS) was used as previously described. [27]

A visual analogue scale (VAS) 10cm line was used to assess overall allergic symptoms at each time point. 0 indicated no symptoms and 10 indicated severe symptoms that are hard to tolerate. For a more detailed description of clinical measurements please refer to the online supplements.

2.7. Statistical Analysis

Statistical analysis of the collected data was performed using R version 3.5 and GraphPad prism (GraphPad Prism 7, La Jolla, California, USA). For a detailed description of the statistical methodology please refer to the online repository.

2.8. Definition of IL-13 Responder Status and Clinical LAR

Previous work has shown that some allergic individuals develop high levels of IL-13 in the LAR while others do not. [3] We also detected this pattern and to investigate this group further we split the provoked cohort into IL-13 Responders ($IL\text{-}13^{R}$) and non-Responders ($IL\text{-}13^{NR}$). $IL\text{-}13^{R}$ were defined as those having increases in IL-13 during the LAR (2-24 hours) at one or more time point/s above the 90^{th} centile of the placebo group. Those in the $IL\text{-}13^{NR}$ group failed to meet this criteria.

Clinically, we defined nasal LAR based on the work by Soliman and Kim et al. [28, 29] as follows: Participants were defined as experiencing a LAR if they showed a ≥25% decrease in their nasal airflow as measured by PNIF and an increase in the nasal obstruction symptom parameter of the TNSS from baseline at 6 hours post provocation.

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3. RESULTS

3.1. Study design and subjects

At the baseline visit (V_B, Figure 1A), participants were randomly allocated to receive provocation with BPE (n=20) or placebo (n=10) on three consecutive days (V_1-V_3, Figure 1A, Table 1). On days one and three of nasal challenge, clinical parameters (PNIF, VAS, TNSS) were assessed and nasosorption samples were collected before and at selected time points up to 24h after provocation. All 30 participants completed the study (Figure 1B).

3.2. Clinical response to nasal challenge with birch pollen extract

Clinical symptoms in response to nasal challenge were assessed by the PNIF as well as by assessing symptoms using TNSS and VAS. Following nasal provocation the mean area under the curve (AUC) of PNIF was significantly reduced in the BPE as compared to the placebo group (P=0.002) (Figure 2A). PNIF values in BPE provoked participants followed similar kinetics during both days of measurement (Figure 2A). Individuals in the BPE group also reported a significantly higher symptom burden assessed by VAS (P<0.0001) (Figure 2B) and TNSS (P<0.0001) (Figure 2C). BPE-provoked individuals reported significantly higher scores for all four TNSS parameters: nasal obstruction (P<0.0001), rhinorrhoea (P<0.0001), nasal itching (P=0.0001) and sneezing (P<0.0001) (Figure 2 D-G). The symptom burden of the VAS and TNSS in the LAR (2-24h) in all birch provoked participants represented 27.7% and 29.1%, respectively, of the whole symptom burden. The proportion of the symptom burden in the LAR was higher for nasal obstruction (32.8%) and rhinorrhoea (33.1%) and lower for nasal itch (22.1%) and sneezing (14.5%).

3.3. Increased type two cytokines in the nasal mucosa after nasal provocation with birch pollen extract

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We observed clear increases in the type two cytokines, IL-4 and IL-5 in the BPE group on day one and three of challenge (Figure 3A). AUC values, accounting for the overall response during both 24h sampling time courses, of IL-4 (P=0.0311) and IL-5 (P=0.0002), were found to be significantly higher in BPE-provoked participants (Table 2). Secondly, we also analysed the secretion of pro-inflammatory cytokines such as IL-1β, IL-18 and IL-6 in MLF. IL-1β was induced earlier and stronger in BPE participants at visit one following provocation (Figure 3B). In the case of IL-18, BPE provocation triggered a decrease in concentrations at visit one only. IL-6 secretion was induced 40 minutes after BPE provocation at visit one and to a minor extent at visit three (Figure 3B). When analysing the overall response of these cytokines, only IL-6 (P=0.0490) and IL-18 (P=0.0276) were significantly different (Table 2). Thirdly, we investigated alarmins (TSLP, IL-25 and IL-33) and sST2 (a soluble secreted decoy form of the IL-33 receptor). TSLP and sST2 were induced in BPE provoked participants at provocation days one and three, showing higher concentration in birch provoked participants (Figure 3C and Table 2; TSLP: P=0.1829, sST2: P=0.0045)). No differences in IL-33 (P=0.7557) and IL-25 (P=0.8458) concentrations were found between provoked and placebo participants (Figure 3C, Table 2 and Figure E1 in the Online Repository). Lastly, the myeloid cell-associated proteins EDN, MCP-1 and tryptase were induced after BPE provocation at both visits (Figure 3D). Whilst tryptase was induced immediately after provocation, EDN and MCP were induced one hour after allergen exposure. Furthermore, AUC values in EDN (P=0.0001) and tryptase (P=0.0244), but not MCP-1 (P=0.2865), were significantly higher in BPE-provoked participants (Table 2). For the other measured cytokines, no significant differences were observed (Table 2, Figure E1 and E2 in the Online Repository). These results indicated a robust mast cell-driven EAR, with a more heterogeneous LAR dominated by type-two cytokines.

3.4. Consecutive nasal allergen provocation and repetitive sampling induces distinct nasal cytokine secretion profiles.

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We noted that repetitive nasal sampling performed during the time courses on the provocation days induced the secretion of IL-16, MCP-2 and TNF-α in both groups (Figure E2A in the Online Repository). The concentration of these three cytokines displayed a progressively higher level over time and peaked well above the baseline values 8h after provocation in both visits. This secretion profile was also observed for other cytokines such as IL-17A and IL-22 (Figure E1 in the Online Repository). This pattern may be a result of repetitive sampling or diurnal variation of nasal cytokines.

Secondly, some mediators such as eotaxin, MPO and TGF-β displayed a marked decline in concentration below baseline values right after challenge in both visits and groups (Figure E2B in the Online Repository). The observed decline took place during the intensive sampling phase before returning to baseline levels. This trend was also observed for some other cytokines such as BAFF, IL-7, IL-8, IL-12p70, Gro-α and NGAL (Figure E1 in the Online Repository) as well as IL-1β (Figure 3B).

With regards to consecutive BPE challenge we noted two different effects on some of the cytokine secretion profiles: GM-CSF (Figure E2C in the Online Repository) as well as IL-4, IL-5, IL-6, IL-13, TSLP and tryptase (Figure 3) reached lower peak concentrations after the third provocation compared to the first. On the contrary, EDN content displayed higher peak concentrations on day 3 (Figure 3D).

3.5. IL-13 responders show an enhanced clinical late allergic response with significantly increased levels of TSLP and type two cytokine levels after nasal allergen challenge not seen in non-responders despite similar early allergic response reactions

We grouped "IL-13 responders (IL-13^{R})" as those participants challenged with birch extract whose IL-13 concentrations were above the 90^{th} centile of the placebo group for at least one time point between 2h and 24h during the first provocation time course (90^{th} centile at: 2h=6.1 pg/ml, 4h=7.1 pg/ml, 6h=10.7 pg/ml, 8h=8.5 pg/ml, 24h=8.9 pg/ml). This resulted in eight

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participants being classified as IL-13^{R} and 12 as IL-13 non-responders (IL-13^{NR}) (Figure 4A). Participants classified as IL-13^{R} experienced a secondary drop in PNIF not seen in their IL-13^{NR} counterparts reaching significance at 6h vs IL-13^{NR} (P=0.0185) and placebo (P=0.0018) (Figure 4B and C, respectively). All IL-13^{R} experienced a clinical LAR (see methods). Only one patient in the IL-13^{NR} group also showed a clinical LAR according to our definition at 6 hours, but this patient showed a very slow recovery in the PNIF over the whole time course and no secondary drop in nasal airflow (data not shown). Furthermore, a higher overall symptom burden was seen in the VAS and TNSS of IL-13^{R} but did not reach significance against IL-13^{NR} participants (Figure 4B and C). Additionally, IL-13^{R} participants reported significantly more nasal obstruction than IL-13^{NR} and maintained significance against placebo in rhinorrhea, nasal itch and sneezing in the LAR (Figure 4B and C).

Next, we analysed nasal cytokine responses in IL-13^{R}, IL-13^{NR} and placebo participants. IL-5, IL-6, IL-22, sST2, TSLP, GM-CSF and MCP-1 presented significantly higher concentrations in IL-13^{R} than in IL-13^{NR} participants (all $P<0.05$, Figures 4D, E and Figure E3 in the online repository). Remarkably, these nasal cytokine responses in the LAR of IL-13^{NR} participants were not significantly different than in the placebo group. No significant differences were found in IL-4 and IL-33 responses (Figure 4D, E) between IL-13^{R} and IL-13^{NR}. Responses in the EAR in both groups were very similar (Figure E4 in the Online Repository). Interestingly, the baseline concentration of IL-5 and MCP-1 were significantly higher in IL-13^{R} vs IL-13^{NR} participants (Table E2 in the Online Repository). For time course response curves in IL-13^{R}, IL-13^{NR} and placebo groups on both days please refer to Figure E5-E7 in the Online Repository.

Data from all 30 participants were included in a correlation matrix analysis of selected parameters (AUC values of provocation day 1) during both EAR and LAR. In the EAR, tryptase showed a strong correlation with nasal obstruction, VAS and TNSS and PNIF (Figure

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5A, Table E3 in the Online Repository). Importantly clinical symptoms of the LAR as measured by VAS and TNSS (especially the item nasal obstruction) were associated with secretion of type 2 cytokines IL-4, IL-5 and IL-13 as well as with TSLP, sST2, EDN and IL-6 secretion (Figure 5B, Table E4 in the Online Repository). Together, these results indicated that a cohort of participants have robust nasal IL-13 responses in the LAR following birch challenge, and this is associated with nasal obstruction and a diverse inflammatory response in the nasal mucosa.

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4. DISCUSSION

This study is the first to thoroughly characterise nasal cytokine responses to controlled birch pollen exposure and link them to the symptomatic reactions with an in-depth characterisation of the LAR. Firstly, in the EAR, we showed that nasal tryptase and sST2 increased within the first two hours after allergen contact and correlated significantly with TNSS and VAS. During the LAR (> 2 hours after provocation) we observed increased levels of IL-4, IL-5, IL-6, sST2, TSLP and EDN in BPE- versus placebo-provoked participants. We also showed that the LAR accounts for approximately 30% of the total symptom burden after nasal provocation in susceptible participants. Interestingly, if grouped by IL-13 responses in the LAR, IL-13^{R} showed strong late phase reactions which were much lower or absent in IL-13^{NR}.

While several studies have investigated the effect of grass pollen or cat allergen on nasal cytokine levels, [3, 6, 7, 9, 30, 31] studies on the impact of birch pollen on nasal cytokine profiles are scarce and assessed only few selected mediators. [32] Further study is important as it has been estimated that 8-16% of the general population in Europe is sensitised to birch pollen. [33] Here we analysed a large panel of 33 cytokines in nasal MLF after a three day consecutive challenge with BPE. Importantly, a placebo control group allowed us to clearly detect allergen-specific responses. The allergen concentration applied was previously shown to induce a significant increase in major birch pollen allergen Bet v 1 specific IgE levels. [2, 34] In accordance with previous provocation studies using different allergens, we observed an early increase in tryptase and sST2. Furthermore, the late allergic response was characterized by a rise in type two cytokines (IL-4, IL-5 and IL-13), pro-inflammatory cytokines such as IL-1β and IL-6 as well as the eosinophil degranulation product EDN. [3, 6, 7, 8, 30] In addition, using a highly sensitive assay, we identified a significant rise of the alarmin TSLP after the first allergen challenge which previous studies were unable to detect. [7, 17]

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Using a provocation schedule of BPE challenges over three consecutive days, we observed a trend towards a reduced response on day 3 of provocation with the exception of EDN. This intense provocation schedule was chosen as firstly it mimics natural birch pollen season [35] and secondly as no immediate increase in IgE levels within this short period of time was to be expected that may have altered nasal cytokine levels. This trend towards reduced response on day three is in contrast to previous studies, which reported a priming of an immune response and thus an increase in cytokine levels after several challenges. [31, 36, 37, 38, 39] Potential explanations for this discrepancy lie firstly in the use of birch pollen as a model allergen, since it does not have high protease activity. [37, 38] Furthermore with regards to the late phase response, we did not observe the response to be more pronounced or more participants suffering from late phase responses on day three (Figures E4-E6 in the online repository). This observation is in accordance with previous findings as it seems that priming of LAR requires more time : Using a low-dose allergen challenge for five days, Orban et al found significant priming of the LAR only on day 11 after the first challenge [31], but not on day 3. Thus, it is conceivable that a boosting of the allergen-specific systemic immune response, which occurs as early as 1-2 weeks after the nasal challenge [2], may have been responsible for the priming effect observed by Orban et al, whilst the three challenges in short intervals in our study may have led to an exhaustion of available immune cells by day three.

Additionally using a placebo group we were also able to assess the effect of daily repetitive sampling on the secretion of nasal cytokines. Firstly, some cytokine concentrations (i.e. IL-16, MCP-2 and TNF-α) rose with time regardless of the nature of provocation indicating that their release was possibly triggered mechanically. [40] A second group of cytokines showed a strong decrease within 1 hour after provocation (i.e. eotaxin, MPO and TGF-β). For the majority of the cytokines we hypothesize that this occurred due to the intense sampling within the first hour “washing out” cytokines present at homeostasis followed by a return to baseline as the

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383 sampling intensity decreased. This washout effect may also explain why we do not observe
384 increased levels of eotaxin or eotaxin-3 as previously described. [7, 36, 41, 42]

385 In this study in susceptible participants, we considered the nasal LAR to be occurring between
386 2-8 hours. Although some publications have defined LAR as occurring 6-7 hours after
387 allergen challenge [28, 29] there is no consensus on the optimal time-point for measuring nasal
388 LAR and our chosen timeframe reflects the timeframes chosen by studies using similar
389 challenge models [3, 11, 31, 43, 44] whereas some of those choosing later times have had different
390 challenge models e.g. environmental challenge chamber. [29]

391 Based on previous reports suggesting an association of IL-13 levels with the occurrence of a
392 late phase response, we grouped our participants based on their IL-13 response status in the
393 LAR after nasal provocation into IL-13^{R} and IL-13^{NR}. (3, 45) Indeed using these objective
394 and subjective clinical parameters, we demonstrated that the IL-13^{R} participants had
395 significant elevations of type 2 cytokines over placebo and IL-13^{NR} and showed the typical
396 symptoms of a late phase response. [10, 11, 12] This was observed in 8 out of 20 of our birch
397 pollen-provoked participants which is in accordance with reports suggesting 50% of allergic
398 individuals experience a LAR. [11, 12, 13, 14, 15] IL-13 has been shown to have distinct roles in
399 allergic diseases despite sharing a receptor (IL-4R) with IL-4. IL-13 plays a key role in the
400 pathological features of disease, such as mucus production, airway hypersensitivity and
401 collagen distribution [46, 47] which would further support the theory that individuals susceptible
402 to significant IL-13 release experience a symptomatic LAR.

403 In terms of other cytokine responses, we observed a significant rise in IL-5, IL-6, TSLP, GM-
404 CSF, sST2 and MCP-1 in the IL-13^{R} group only. Local cytokine responses are of course
405 related to the cellular environment and it has been described that recruitment of Th2 CD4+
406 cells occurs in the LAR. [48] In those susceptible to the LAR their presence would result in
407 high levels of IL-5 and IL-13 leading to recruitment of eosinophils and basophils. [48]

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Activated eosinophils would contribute to a further increase of IL-5 production as well as EDN leading to local inflammation and epithelial damage, [10, 49] releasing TSLP. [50] The alarmin TSLP is known to trigger the activation of ILC2s, Th2 cells and eosinophils. [50] Here we detected high levels of TSLP already 4h after provocation and thus it may provide a positive feedback loop for IL-13 release, which peaks at 6-8h. Interestingly, an sST2 peak in the LAR was only present in IL-13^{R} participants. sST2 in the LAR has been theorised to be of basophil origin which also release histamine. [51, 52] sST2 acts as a decoy receptor for IL-33 thus inhibiting binding of IL-33 to its cellular receptors. It has been shown to be inversely correlated with symptom severity during peak season. [51] Furthermore, histamine secreted by basophils in the LAR has been shown to induce IL-6 release from monocytes [53] which in turn has been shown in the context of allergic rhinitis to inhibit ILC2 function, [54] a known source of type two cytokines. IL-6 is also known to modulate eosinophilia [55] thereby suggesting an immunomodulatory function in regulating excessive type two cytokine production. IL-6 also showed a significant increase in the IL-13^{R} group only, thus, it may be speculated that ST2 and IL-6 are secreted in IL-13^{R} participants in LAR to counteract the strong type-2 biased inflammatory environment.

No study to date has identified individuals susceptible to LAR in allergic rhinitis at baseline and then gone on to study these individuals at a cellular and molecular level. Our study paves the way to do this as remarkably, in IL-13^{R} participants we observed not only significantly increased levels of MCP-1 and IL-5 after allergen challenge, but also elevated baseline levels in comparison to IL-13^{NR} participants. MCP-1 is a potent chemoattractant for monocytes and in the context of allergic rhinitis has been shown to be important for the recruitment of macrophages, T cells, eosinophils and basophils. [56, 57] Thus, it is conceivable that the elevated baseline MCP-1 levels in the IL-13^{R} participants could predispose them to stronger inflammatory responses including recruitment of IL-13 producing T cells in the LAR. In this respect, polymorphisms in the gene regulatory region of MCP-1 have been associated with

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asthma susceptibility and severity. [58] Furthermore, administration of anti-IL5 antibody prior to allergen challenge in a murine asthma model completely abolished LAR and influx of eosinophils into the lung. [59] However our findings need to be interpreted with caution due to the high number of parameters tested and the fact these biomarkers only just reached statistical significance.

Being able to identify individuals who are susceptible to LAR versus those who experience mainly strong EAR symptoms in the absence of LAR could be important in treatment stratification. In this context the choice of treatment should carefully be evaluated based on the following considerations: Antihistamines such as cetirizine though very effective in combating the symptoms generated as a result of mast cell degranulation have shown no effect on the symptoms in the LAR. [18] In contrast, pre-treatment with glucocorticoids effectively alleviates symptoms of the LAR [19] but not in the EAR. With new biological therapies on the market there could be potential to apply these treatments to individuals suffering from severe late phase reactions. For instance, mepolizumab, an anti-IL-5 monoclonal antibody indicated for severe asthma with eosinophilia, could be useful in individuals suffering from LAR as it would block IL-5, which we have shown is actively secreted during this phase. Indeed, administration of anti-IL-5 antibody prior to allergen challenge in a murine asthma model completely abolished LAR and influx of eosinophils into the lung. However, so far this has not translated into humans where the anti-IL-5 antibody mepolizumab and the anti-IL-5 receptor alpha antibody benralizumab showed no effect in reducing LAR in the skin or lung respectively, despite significantly reducing skin and sputum eosinophil counts [60, 61]. To our knowledge it has not yet been investigated if anti IL-5 treatment could have an effect on allergic rhinitis patients suffering from LAR if applied prior to allergen exposure. Additionally, although anti-IL-13 therapy showed no overall benefit in a trial in allergic rhinitis, a subgroup analysis of those suffering from high IL-13 secretion in the LAR showed a reduction in symptoms, highlighting the potential benefit and need for good

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patient selection. [45] Furthermore, the only available curative therapy, allergen immunotherapy, should be considered also especially for LAR patients as it does not only significantly reduce bronchial and cutaneous LAR in allergic patients, [62, 63] but also leads to diminished nasal IL-4, IL-5 and IL-13 production upon allergic provocation. [8] Therefore, in the future and if our data can be confirmed in a larger population, clear treatment recommendations would be available based on patients response to IL-13 in the late phase after allergen provocation. Additionally although the baseline data needs to be interpreted with caution, baseline nasal MCP-1 and IL-5 levels could also identify individuals susceptible to LAR.

One limitation of our study is that we analysed only MLFs but no tissue derived samples. However, as our main goal was to understand the mediator kinetics underlying the allergen-specific mucosal response, it was not possible to collect tissue samples from the same participants at multiple time points as the tissue injury caused by the collection itself would have significantly altered the mediator profile in the MLF. Additionally for measurements of nasal flow we did not use rhinomanometry but PNIF. Nonetheless due to our intense sampling schedule rhinomanometry would not have been possible and the PNIF is a widely accepted device which has been used in many clinical trials and has been shown to be comparable to rhinomanometry in distinguishing between healthy and pathologic states. [26, 64]

In summary, our data provide insights into the cytokine responses underlying early and late allergic responses to birch pollen exposure. We demonstrated that participants who experience a symptomatic LAR show markedly elevated levels of cytokines associated with type two responses as well as sST2, EDN and MCP-1. Our work suggests that the measurement of allergen-induced IL-13 in nasal fluids can be used to identify individuals with LAR to provide them with more personalised treatment for their symptoms. Based on data from this pilot study, a time point between 4-8 hours after nasal allergen provocation would

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485 be well suited for nasal sampling with the clear cut-off level remaining to be determined in
486 future trials involving more participants.

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6. Figure legends:

Figure. 1: Overview of Study Design and Sampling Time course. (a) Birch pollen allergic participants were screened (V_S) and participants meeting all inclusion criteria attended a baseline visit (V_B) taking place at a maximum of two weeks before the start of the study. At V_1, V_2 and V_3, participants underwent nasal challenge (NC) with either birch pollen extract or normal saline solution (=placebo). On provocation days one (V_1) and three (V_3) study participants underwent nasal sampling immediately before provocation (baseline: BL) and 10, 20, 40 min and 1, 2, 4, 6, 8 and 24h after NC. BAT = basophil activation test, PNIF = peak nasal inspiratory flow, SPT= skin prick test, TNSS = total nasal symptom score, VAS = visual analogue scale, **(b)** flow chart of participants who were assessed, randomized and analysed.

Table 1: Demographic and serological data at baseline in study groups. Ranges, medians and standard deviations are displayed where appropriate. BPE = birch pollen extract. P-values are indicated.

Figure 2: Clinical response to nasal provocation with birch pollen extract or placebo. Participants undergoing nasal provocation with birch pollen extract (red, n= 20) and those being provoked with placebo (grey, n=10). **(A-G)** Left panels show time since provocation (x-axes) against **(A)** Mean fold change in peak nasal inspiratory flow (PNIF) (y-axis; Time point PNIF/Baseline PNIF), **(B)** Mean visual analogue scale (VAS) score (y-axis, cm), **(C)** Mean total nasal symptom score (TNSS) (y-axis) and **(D-G)** Breakdown of the individual components of the TNSS (y-axis): **(D)** Mean symptom score of nasal obstruction**, (E)** rhinorrhea, **(F)** nasal itching and **(G)** sneezing. Right panels show the mean area under the curve (AUC) values for the respective graphs and groups for provocation day 1 and 3 combined. Error bars represent standard error of the mean. Stars represent statistically significant differences between AUC values (*: P≤0.05, **: P≤0.01, ***: P≤0.001, ****P≤0.0001).

Table 2: Cytokine release across visits V1 and V3 in participants provoked with birch pollen extract or placebo. Mean area under the curve (AUC) cytokine values, standard deviation (SD) and significant differences between birch pollen extract (BPE) and placebo provoked participants (P value) are displayed. Cytokines with significant differences (P≤0.05) are represented in bold.

Figure 3: Selected mean mediator responses in nasal secretion samples collected at visit V1 and V3 in participants undergoing nasal provocation with birch pollen extract (red, n= 20) vs those being provoked with placebo (grey, n=10). (A-D). Time course response graphs of **(A)** type two cytokines (IL-4, IL-5, IL-13), **(B)** pro-inflammatory cytokines (IL-1β, IL-18, IL-6), **(C)** alarmins and their soluble receptors (IL-33, sST2,

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TSLP), **(D)** mast cell, eosinophil and monocyte mediators (EDN, MCP-1, tryptase) as mean concentration (y-axis, pg/ml) over time since provocation (x-axis). Error bars represent the standard error of the mean.

Figure 4: Selected clinical and mean mediator responses in nasal secretions collected at visit V1 in participants with significant IL-13 responses in the LAR (IL-13 responders (IL-13^{R}), green, n= 8) vs those showing no IL-13 response (IL-13 non-responders (IL-13^{NR}), blue, n= 12) vs those being provoked with placebo (normal saline) (grey, n=10). (A) Grouping strategy for determining IL-13 status; IL-13 (y-axis, pg/ml) over time (x-axis). IL-13 status was defined based on whether participants had their IL-13 levels rise above the 90th centile of the placebo group (black dashed line = placebo 90th centile, black line = placebo mean) at, at least 1 time point within the late phase (defined as 2-24h highlighted by red dotted lines which are represented slightly out of position to increase graphical clarity). **(B)** Mean area under the curve (AUC) values in the late phase (2-24h) (y-axis) and **(C)** time course graphs (x-axis: time; y-axis as indicated in individual graphs) for the following clinical parameters at visit V1 in IL-13^{R} vs IL-13^{NR} vs placebo provoked participants: Peak nasal inspiratory flow (PNIF), visual analogue scale (VAS), total nasal symptom score (TNSS) and breakdown of the individual components of the TNSS (nasal obstruction, rhinorrhea, nasal itching, and sneezing) **(B)** Bars or **(C)** points represent averages and error bars represent the standard error of the mean. **(D, E): (D)** Mean AUC mediator values in the late phase (2-24h) (y-axis) and **(E)** Time course response graphs (x-axis: time; y-axis, pg/ml) for selected cytokines (IL-4, IL-5, IL-6, IL-13, IL-33, sST2, TSLP, EDN, MCP-1) for visit V1 in IL-13^{R} vs IL-13^{NR} vs placebo). **(D)** Bars or **(E)** points represent averages and error bars represent the standard error of the mean. Where appropriate, stars were used to represent statistically significant differences (*: P≤0.05, **: P≤0.01, ***: P≤0.001, ****P≤0.0001).

Figure 5: Correlation matrices of selected mediators and clinical parameters in the early (0-1h) and late phase (2-8h) after nasal provocation. (A, B) Correlation matrix of selected mediators and clinical parameters in the **(A)** early phase (time points 10, 20, 40 min and 1h) or **(B)** late phase (2, 4, 6, 8h) after nasal provocation during visit V1. Only those statistically significant correlations (P≤0.05) have a dot shown in the matrix. Intensity of colour represents the strength of either a positive (blue) or a negative (red) correlation according to Spearman's rank correlation coefficient. For correlation calculations all values from all 30 participants were included. Boxes represent clustering analysis.

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		Placebo Group	BPE Group	P value
Sex	*M/F*	2/8	8/12	n/a
Age	*Mean ± SD*	30.70 ± 6.86	30.20 ± 9.06	08677
	Range	24-43	20-49	
Bet v 1 sIgE (kUA/l)	*Mean ± SD*	22.06 ± 20.18	28.19 ± 33.07	0.5356
	Range	4.1-69	1.5-110	
Total IgE (kU/l)	*Mean ± SD*	161.68 ± 82.23	325 ± 471.88	0.1476
	Range	71.3-294	9.8-1728	

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	BPE mean AUC	*Placebo mean AUC*	*BPE SD*	*Placebo SD*	*P Value*
IL-1α	808.6	954.4	320.8	507.2	0.5018
IL-1β	311.1	311.5	191.5	185.1	0.9828
IL-4	**338.2**	**195.5**	**191.7**	**104.7**	**0.0311**
IL-5	**1132.8**	**112.0**	**1511.4**	**95.6**	**0.0002**
IL-6	**2622.0**	**1613.6**	**1711.8**	**1651.9**	**0.0490**
IL-7	3377.9	3001.1	1711.5	1549.6	0.8798
IL-8	99728.6	86939.8	46668.9	40672.9	0.4745
IL-10	390.1	261.5	236.6	162.5	0.1980
IL-12p70	2126.8	2021.3	900.7	993.2	0.8458
IL-13	125.7	108.9	70.1	13.6	0.9747
IL-16	22365.3	16839.5	10453.6	11158.9	0.1307
IL-17A	5883.3	5675.7	3614.0	2780.8	0.7787
IL-18	**39492.7**	**50504.7**	**9736.4**	**12416.8**	**0.0276**
IL-21	1998.4	2238.7	475.6	752.7	0.3524
IL-22	151.0	132.7	200.4	82.5	0.6494
IL-25	346.4	345.5	142.7	101.4	0.8458
IL-33	657.6	699.6	375.6	381.9	0.7457
APRIL	49760.1	37191.3	26231.3	25812.3	0.1829
BAFF	4681.0	4158.7	2232.5	2437.5	0.4745
EDN	**18416.3**	**2409.8**	**14763.3**	**2031.2**	**0.0001**
Eotaxin	14892.4	12082.5	8189.1	7326.2	0.3504
GM-CSF	25.3	6.5	62.1	6.1	0.0870
GRO-α	397.1	361.0	339.2	366.5	0.7132
IFN-γ	8216.2	12082.5	5242.5	5077.3	0.9483
MCP-1	879.6	580.4	690.8	328.4	0.2865
MCP-2	1.2	0.8	1.4	1.0	0.8766
MPO	9579.6	10371.8	5344.1	6974.4	0.8121
NGAL	19044.1	20836.1	10983.0	13737.8	0.6496
sST2	**29448.6**	**15154.0**	**14325.4**	**9583.8**	**0.0045**
TGF-β	25856.8	31891.4	22393.6	29473.2	0.5300
TNF-α	441.6	323.9	250.3	134.8	0.3069
Tryptase	**206.4**	**86.8**	**159.3**	**101.6**	**0.0244**
TSLP	883.8	572.0	543.5	200.8	0.1829

Table 2_Campion et al.

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	IL-18	IL-33	sST2	Tryptase	PNIF	VAS	TNSS	Nasal Obstruction	Rhinorrhoea	Sneezing	Nasal Itch
IL-18	0	0.053438	0.034832	0.136391	0.029753	0.00233	0.007559	0.004679	0.051299	0.026684	0.034299
IL-33	0.053438	0	0.406223	0.858266	0.858856	0.801781	0.720353	0.500134	0.602769	0.901734	0.980304
sST2	0.034832	0.406223	0	0.003757	0.007307	0.011334	0.002643	0.002145	0.040362	0.00182	0.009444
Tryptase	0.136391	0.858266	0.003757	0	7.85E-05	1.41E-05	0.000308	7.00E-06	0.021654	0.010804	0.03404
PNIF	0.029753	0.858856	0.007307	7.85E-05	0	5.66E-05	0.001454	0.000117	0.019237	0.008516	0.090767
VAS	0.00233	0.801781	0.011334	1.41E-05	5.66E-05	0	6.82E-13	3.65E-11	9.52E-08	4.18E-05	3.67E-05
TNSS	0.007559	0.720353	0.002643	0.000308	0.001454	6.82E-13	0	9.57E-13	1.55E-08	4.90E-08	6.59E-07
Nasal Obstruction	0.004679	0.500134	0.002145	7.00E-06	0.000117	3.65E-11	9.57E-13	0	3.20E-06	6.54E-06	0.000133
Rhinorrhoea	0.051299	0.602769	0.040362	0.021654	0.019237	9.52E-08	1.55E-08	3.20E-06	0	0.000321	0.000877
Sneezing	0.026684	0.901734	0.00182	0.010804	0.008516	4.18E-05	4.90E-08	6.54E-06	0.000321	0	0.000162
Nasal Itch	0.034299	0.980304	0.009444	0.03404	0.090767	3.67E-05	6.59E-07	0.000133	0.000877	0.000162	0

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Table E3_Campion et al.

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	IL-4	IL-5	IL-6	IL-13	IL-33	EDN	MCP-1	sST2	Tryptase	TSLP	PNIF	VAS	TNSS	Nasal Obstruction	Rhinorrhoea
IL-4	0	0.058309	0.027196	0.690287	0.6047	0.000138	0.181627	0.076301	0.297317	0.350749	0.025277	0.001511	0.022818	0.005456	0.133105
IL-5	0.058309	0	7.54E-05	0.000161	0.841714	2.75E-05	0.003494	9.76E-05	0.053557	6.91E-06	0.001047	2.64E-05	3.79E-05	1.79E-05	0.001307
IL-6	0.027196	7.54E-05	0	0.003097	0.060699	0.00622	0.00071	0.000438	0.018626	0.000208	0.002348	0.012627	0.006486	0.002786	0.042401
IL-13	0.690287	0.000161	0.003097	0	0.857384	0.033736	0.089524	0.00613	0.076565	3.50E-05	0.165961	0.074736	0.090775	0.102542	0.016334
IL-33	0.6047	0.841714	0.060699	0.857384	0	0.945692	0.102992	0.738548	0.000748	0.58832	0.051	0.601696	0.467441	0.351485	0.799145
EDN	0.000138	2.75E-05	0.00622	0.033736	0.945692	0	0.24261	0.002852	0.183991	0.002571	0.007094	0.000177	0.001824	0.000576	0.079318
MCP-1	0.181627	0.003494	0.00071	0.089524	0.102992	0.24261	0	0.007283	0.023343	0.048899	0.015875	0.290042	0.563157	0.135341	0.972196
sST2	0.076301	9.76E-05	0.000438	0.00613	0.738548	0.002852	0.007283	0	0.034918	6.15E-06	0.054639	0.033106	0.04724	0.001143	0.540953
Tryptase	0.297317	0.053557	0.018626	0.076565	0.000748	0.183991	0.023343	0.034918	0	0.263508	0.036459	0.359696	0.200076	0.095815	0.834196
TSLP	0.350749	6.91E-06	0.000208	3.50E-05	0.58832	0.002571	0.048899	6.15E-06	0.263508	0	0.058309	0.014221	0.008153	0.003528	0.027574
PNIF	0.025277	0.001047	0.002348	0.165961	0.051	0.007094	0.015875	0.054639	0.036459	0.058309	0	0.018586	0.045619	0.031609	0.306555
VAS	0.001511	2.64E-05	0.012627	0.074736	0.601696	0.000177	0.290042	0.033106	0.359696	0.014221	0.018586	0	4.54E-13	1.08E-10	2.03E-05
TNSS	0.022818	3.79E-05	0.006486	0.090775	0.467441	0.001824	0.563157	0.04724	0.200076	0.008153	0.045619	4.54E-13	0	1.26E-13	1.83E-07
Nasal Obstruction	0.005456	1.79E-05	0.002786	0.102542	0.351485	0.000576	0.135341	0.001143	0.095815	0.003528	0.031609	1.08E-10	1.26E-13	0	0.00011
Rhinorrhoea	0.133105	0.001307	0.042401	0.016334	0.799145	0.079318	0.972196	0.540953	0.834196	0.027574	0.306555	2.03E-05	1.83E-07	0.00011	0

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Table E4_Campion et al.

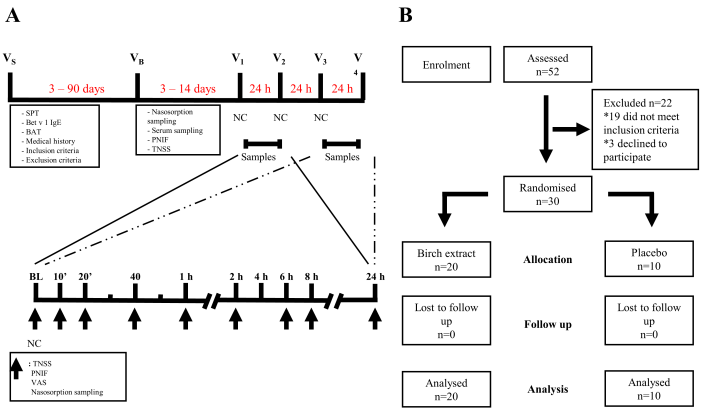

Figure 1_Campion et al.

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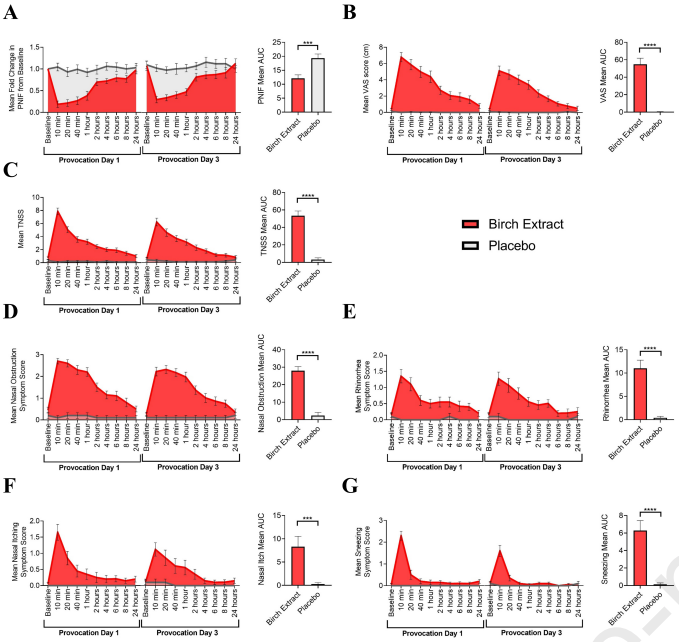

Figure 2_Campion et al.

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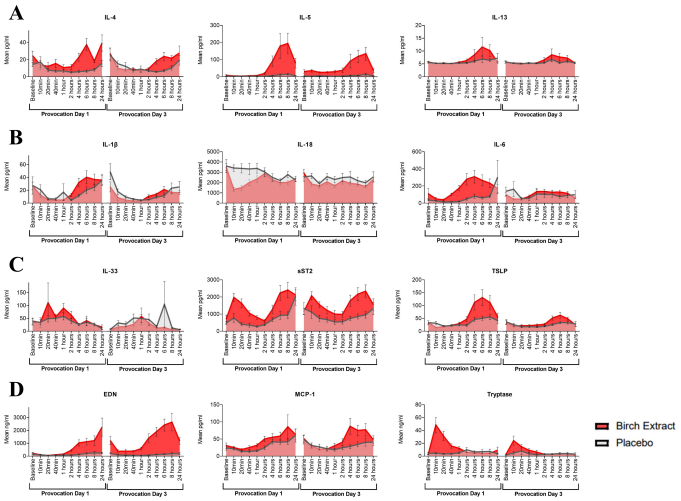

Figure 3_Campion et al.

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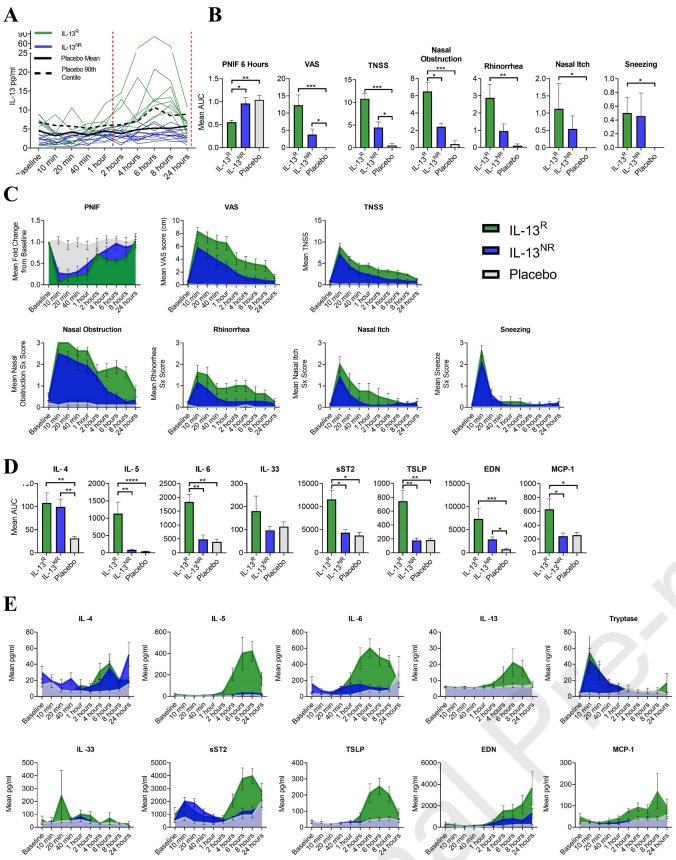

Figure 4_Campion et al.

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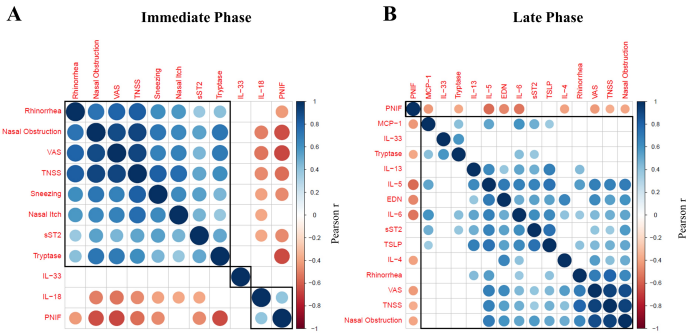

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Figure 5_Campion et al.