

DR AMARYLLIS HACCURIA (Orcid ID : 0000-0001-5779-1729)

DR ANDREI MALINOVSKI (Orcid ID : 0000-0002-4098-7765)

Article type : Research Letter

Increased expression of IL-33 is found in the lower airways of patients with seasonal allergic rhinitis and is not related to natural allergen exposure.

*Amaryllis Haccuria, PhD¹, Alain Van Muylem, PhD¹, Andrei Malinowski, PhD²,
Joanne Rasschaert, PhD³, Myrna Virreira, PhD³, Nicolas Bruffaerts, PhD⁴, Marijke
Hendrickx, PhD⁴, and Alain Michils, PhD¹*

¹Chest Department, Erasme University Hospital, Université Libre de Bruxelles, Brussels, Belgium

²Department of Medical Sciences: Clinical Physiology, Uppsala University, Uppsala, Sweden.

³Laboratoire de Biochimie Métabolique et Osseuse, Université Libre de Bruxelles, Brussels, Belgium

⁴Mycology & Aerobiology, Sciensano, Brussels, Belgium

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/CEA.13819](https://doi.org/10.1111/CEA.13819)

This article is protected by copyright. All rights reserved

Address for reprint requests and other correspondence:

Alain Van Muylem, PhD

Chest Department

CUB Erasme, Université Libre de Bruxelles

808 Route de Lennik

1070 Brussels - Belgium

Phone: +32-2-555-39-85

Fax: +32-2-555-44-11

E-mail: avmuylem@ulb.ac.be

Funding: This study was supported by Fonds Erasme (Convention André Arouète)

Acknowledgements: The authors would like to thank Mr Tom O'Connell from OCPR Brussels for proofreading the manuscript.

Keywords: rhinitis, basic immunology, epithelium, IL-33, united airway disease, induced sputum.

Clinical implications: Our study shows that the so-called 'one airway, one disease' concept linking allergic asthma and allergic rhinitis applies to lower airway epithelial dysfunction.

Running title: IL-33 is released in lower airways of allergic rhinitis patients without concomitant asthma, independently from natural variation in allergen exposure.

Abstract: The IL-33 pathway involved in the development of type-2 airway inflammation is activated in patients with allergic asthma. According to the "one airway, one disease" concept, the IL-33 pathway should also be activated in the airways of patients suffering from allergic rhinitis. **Methods** We compared the levels of IL-33 and its mRNA precursor, in induced sputum of patients suffering from seasonal allergic rhinitis (n=27) with those measured in patients with seasonal allergic asthma (n=23), in atopic asymptomatic subjects (n=9), and healthy controls (n=17), in and out of the pollen season. **Results** Medians IL-33 levels were higher in sputum supernatants of allergic rhinitis (9.4 pg.ml⁻¹) and asthma patients (5.2 pg.ml⁻¹) when compared to controls (0 pg.ml⁻¹; p<0.001) or to atopic asymptomatic subjects (0 pg.ml⁻¹; p=0.006). The same was observed with qPCR. IL-33 levels were similar in and out of pollen seasons in

patients suffering from allergic rhinitis (4.9 pg.ml⁻¹ vs 7.1 pg.ml⁻¹, p=0.256) or allergic asthma patients (3.6 pg.ml⁻¹ vs 3.2 pg.ml⁻¹, p=0.61). Conclusion IL-33 is detectable in the lower airways of patients suffering from allergic rhinitis and to similar levels than in patients suffering from allergic asthma. These levels appear to be independent of atopy per se and of natural variation in allergen exposure. Those data provide evidence of a silent epithelial dysfunction in the airways of patient suffering from allergic rhinitis without asthma, further confirming the «one airway, one disease» theory linking asthma and rhinitis.

Author contribution:

All authors provided critical feedback and helped shape the research, analysis and manuscript.

A Haccuria, A Van Muylem and A Michils conceived, planned the study and wrote the manuscript with input from all authors. M Virreira carried out the experiments, worked out almost all of the technical details under the guidance of J Rascchaert. A Malinovschi contributed to the interpretation of the results and worked on the manuscript. A Van Muylem designed the computational framework, designed the figures and performed statistical data analysis. N Bruffaerts and M Hendrickx carried out the pollen monitoring part of the work (data collection, analysis, manuscript).

Increased expression of IL-33 is found in the lower airways of patients with seasonal allergic rhinitis and is not related to natural allergen exposure.

To the Editor,

IL-33 is a so-called epithelial 'alarmin' released upon specific or non-specific triggers, thereby contributing to the initiation of the allergic cascade. It is a potent cytokine, activating innate type-2 cells (ILC2) and type-2 helper (Th2) cells through IL-1RL1, leading to amplified production of cornerstone type-2 cytokines including IL-4, IL-5, and IL-13 (1). Exerting a local pro-inflammatory action, increased IL-33 levels are found in lower airways of allergic asthma patients (2) causing allergen-induced eosinophilia (3) and in nasal swabs of allergic rhinitis patients (4). However, there is no data on IL-33 expression in the lower airways of patients suffering from allergic rhinitis, while the 'unified airway hypothesis' would predict an activation of the IL-33 pathway in this condition. This is particularly relevant since IL-33 has been shown to be involved in asthma airway remodelling (5). Its potential over-production in the lower airways of patients suffering primarily from allergic rhinitis might, therefore, contribute to them developing asthma.

In addition, allergen exposure is known to trigger IL-33 release in the airways. This has been demonstrated using allergen challenge both in animals (6) and humans (7). However, little is known about the influence of natural allergen exposure on IL-33 release.

In order to investigate whether IL-33 expression rises in the lower airways of patients with allergic rhinitis and to study whether seasonal pollen exposure would be a trigger of IL-33 release, we compared the levels of IL-33 and its mRNA precursor, in induced sputum of 27 patients suffering from seasonal allergic rhinitis, with those measured in 23 patients with seasonal asthma, in nine atopic asymptomatic patients and in 17 healthy controls. Allergic rhinitis and asthma were defined according to ARIA and GINA guidelines, respectively. Asthma diagnosis was based on clinical history of recurrent episodes of wheezing, breathlessness and/or cough associated with significant reversibility to salbutamol or a positive bronchial provocation test to histamine. Seasonal rhinitis was defined as occurrence of sneezing, itchiness and nasal obstruction, without asthma symptoms ever, during spring and/or summer. Healthy controls had no symptoms of rhinitis or asthma or other allergic diseases and they had no positivity in skin prick testing to common aeroallergens, whereas atopic asymptomatic patients disclosed one or more positive skin prick tests without any related asthma or rhinitis symptoms. The study was approved by the local ethics committee of CUB Erasme University Hospital Brussels, Belgium (Ethics Committee Number P2014/088), and all of the involved patients signed consent forms.

Demographic data and clinical characteristics can be seen in table 1. These levels were measured

both during and after the pollen season. Data on ethical issues and statistical analyses are described in the Online Supplementary Information.

FENO levels, spirometry parameters, and sputum induction were performed twice - once out of the pollen season and once during the pollen season, for allergic rhinitis and asthma patients, and once for healthy controls. Real time quantitative PCR for IL-33 was performed on sputum cell pellets, while Enzyme Linked Immunosorbent Assay (ELISA), (R&D Systems, Inc., Minneapolis, MN, USA, detection limits 0.187 to 1.65 pg/ml.) for IL-33 and IL-5 were measured in supernatants of induced sputum samples. Airborne pollen monitoring of (birch trees (*Betula* spp.), grasses (*Poaceae*) and mugwort (*Artemisia* spp.)) was performed with a Hirst-type seven-day volumetric spore trap (Burkard Manufacturing Co., Rickmansworth, UK) located in Brussels. Detailed methods are described in the Online Supplementary Information.

As shown on figure 1, panel A, IL-33 measured by ELISA was significantly higher in sputum supernatants of allergic rhinitis (median 9.4 pg.ml⁻¹, range 0 – 51.1 pg.ml⁻¹) and asthma patients (5.2 pg.ml⁻¹, range 0 – 45.4 pg.ml⁻¹) when compared to atopic asymptomatic subjects (median 0 pg.ml⁻¹, range 0 – 8 pg.ml⁻¹; $p < 0.001$ and $p = 0.006$) and controls (median 0 pg.ml⁻¹, range 0 – 13 pg.ml⁻¹; $p < 0.001$). There were no significant differences between allergic asthma and rhinitis ($p = 0.171$) nor between atopic asymptomatic and control subjects ($p = 0.797$). The same was observed with qPCR for IL-33, as the level of mRNA IL-33 in sputum of allergic rhinitis and asthma patients was significantly higher than those obtained in atopic asymptomatic ($p = 0.003$) and control ($p = 0.009$ and $p = 0.007$) subjects (Figure 1, panel B). A median relative quantification of 8.2 and 13.8 ($p = 0.973$) was observed for rhinitis and asthma patients compared to controls. Atopic asymptomatic subjects did not differ from controls ($p = 0.413$).

There was no significant difference between sputum IL-33 levels during and beyond pollen seasons in patients suffering from allergic rhinitis (median 4.9 pg.ml⁻¹ vs 7.1 pg.ml⁻¹, $p = 0.256$) or allergic asthma patients (median 3.6 pg.ml⁻¹ vs 3.2 pg.ml⁻¹, $p = 0.61$), despite an intense natural pollen exposure during the week before induced sputum procedure (> 80 birch pollen grains, and > 50 grass pollen grains per cubic meter of air) for the majority of patients (17 out of 19 rhinitis patients and 12 out of 17 asthma patients). Data are shown on Figure 1, panel C. This intense pollen exposure elicited a significant increase in inflammatory markers in allergic rhinitis patients. These markers include FENO ($p < 0.001$), sputum eosinophils ($p = 0.007$) and IL-5 ($p = 0.036$).

Our study shows that the IL-33 pathway is activated in the lower airways of patients suffering from allergic rhinitis in the absence of concomitant asthma. In fact, IL-33 levels in induced sputum are similarly increased in patients with seasonal allergic rhinitis or seasonal asthma when compared to healthy controls. This was shown to be the case using either qPCR to assess IL-33 mRNA precursors thought to reflect IL-33 DNA synthesis pathway, or ELISA to determine IL-33 protein levels in the supernatants. Our data show that besides macroscopic abnormalities such as epithelial shedding (8), allergic rhinitis and allergic asthma also share some airway epithelial dysfunction characterised by the release of increased amounts of the alarmin IL-33. Whether this also concerns TSLP, another alarmin often secreted together with IL-33 by airway epithelial cells of asthma patients, (9) has yet to be investigated.

The levels of IL-33 products measured by qPCR or ELISA in sputum of atopic asymptomatic subjects (i.e. subjects with IgE sensitisation to aeroallergens but no related atopic disease) were shown to be low and similar to those observed in healthy controls. Moreover, the increase of IL-33 airway expression in both seasonal allergic rhinitis and asthma was independent of natural variations of allergen exposure. It is therefore likely that the IL-33 pathway involved in type-2 response is activated only in the presence of an active airway disease regardless of atopic status per se or natural allergen exposure. This is in line with a recent study that has documented higher IL-33 levels in nasal fluid samples of patients complaining of seasonal rhinitis symptoms, even without pollen sensitization (10).

Taken together, these data further confirm that the so-called 'unified airway hypothesis' linking asthma and rhinitis (4) also applies to a major epithelial function, i.e. IL-33 production. There could be speculation that IL-33 might be related to aspects of airway dysfunction, such as airway remodelling (5) and/or hyperresponsiveness, in allergic rhinitis. This is currently under investigation and will help us in clarifying the potential clinical impact of our findings.

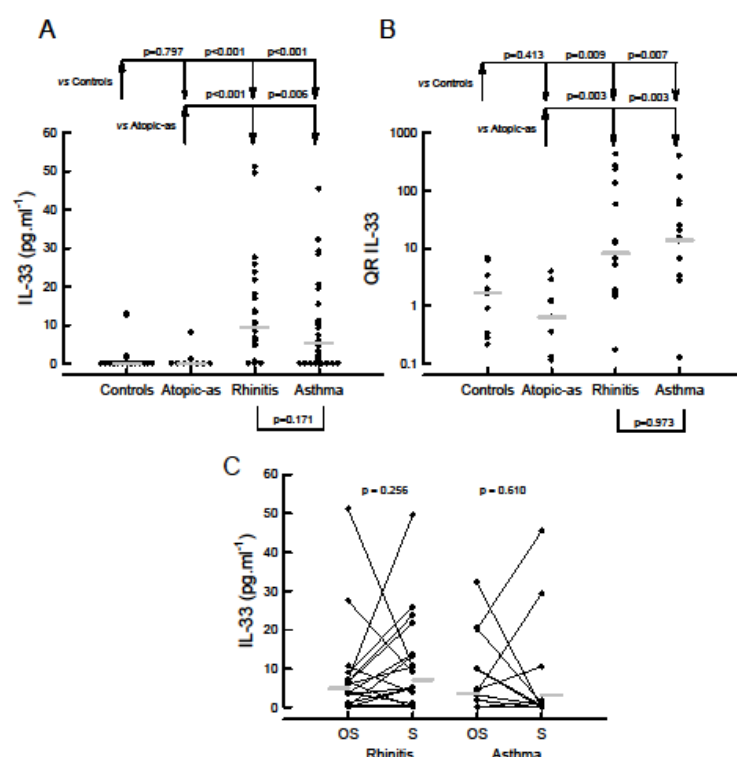


Figure 1: Panel A and B: IL-33 protein (Panel A) and RNA (Panel B) in the study populations (out of pollen season for rhinitis and asthma patients). Panel C: Individual ELISA IL-33 changes between out of pollen season (OS) and pollen season (S) in rhinitis and asthma patients. Grey horizontal bars are the median values.

Table 1: Subjects characteristics and baseline inflammatory markers

Control	Atopic	Rhinitis ^s	Asthma ^s	p
---------	--------	-----------------------	---------------------	---

asymptomatic

	(n=17)	(n=9)	(n=27)	(n=23)	
F/M	11/6	8/1	10/17	7/16	0.007*
Age (year)	34 ± 13	32 ± 13	34 ± 10	35 ± 10	0.589**
FEV ₁ (%pred)	104.9 ± 9.2	100.7 ± 16.5	102.6 ± 11.0	101.9 ± 10.7	0.497**
FENO (ppb)	10.4 [6.8 - 16]	10.3 [5.5 - 19.2]	19.1 [8.9 - 41]	41.2 [21.6 - 78.6]	<0.001**
Cells (10 ⁵ .ml ⁻¹)	26 [7 - 128]	38 [14 - 98]	11 [8 - 41]	14 [3 - 53]	0.002***
Eosinophils (%)	0 [0 - 0]	0 [0 - 0]	1.1 [0 - 10.1]	2.2 [0 - 15.1]	<0.001***
Macrophages (%)	40 [1.9 - 81]	35 [11 - 61]	51 [3 - 83]	55 [5 - 88]	0.670***
Neutrophils (%)	54 [16 - 94]	61 [39 - 98]	45 [0 - 88]	41 [0 - 97]	0.402***
Lymphocytes (%)	2.4 [2.4 - 8.9]	0 [0 - 1.2]	1.8 [2.8 - 34.1]	2.8 [1.8 - 34.1]	0.005***
IL-5 (pg.ml ⁻¹)	0 [0 - 0]	0 [0 - 0]	1.8 [0 - 7.7]	2.9 [0 - 10.0]	<0.001***

§: out of pollen season; *: Exact Fisher test; **: one-way analysis of variance; ***: Kruskal-Wallis test. *Data are presented as mean (m) ± standard deviation (SD) for age and FEV₁; as geometric mean (antilog(m)) [geometric interval] ([antilog(m-SD) – antilog(m+SD)]) for FENO and as median [inter-quartile interval] for cells, eosinophils, macrophages, neutrophils, lymphocytes and IL-5.*

KEY WORDS: allergic rhinitis, allergic asthma, IL-33, natural pollen exposure, sputum induction.

ACKNOWLEDGEMENTS

The authors would like to thank Mr Tom O'Connell from OCPR Brussels for proofreading the manuscript.

CONFLICTS OF INTEREST

Dr. Haccuria reports grants from Chiesi, grants from Astra Zeneca, personal fees from Novartis, personal fees from GSK, non-financial support from TEVA, personal fees from Stallergenes, personal fees from Menarini, grants from Fonds Erasme, during the conduct of the study. Dr. Michils reports grants from Chiesi, grants from Astra Zeneca, personal fees from Chesi, personal fees from Astra Zeneca, personal fees from GSK, personal fees from Novartis, personal fees from Stallergènes, non-financial support from Astra Zeneca, non-financial support from Chiesi, outside the submitted work.

FUNDING INFORMATION

This study was supported by Fonds Erasme (Convention André Arouète)

Amaryllis Haccuria¹

Alain Van Muylem¹

Andreï Malinovschi²

Joanne Rasschaert³

Myrna Virreira³

Nicolas Bruffaerts⁴

Marijke Hendrickx⁴

Alain Michils¹

¹Chest Department, Erasme University Hospital, Université Libre de Bruxelles, Brussels, Belgium

²Department of Medical Sciences: Clinical Physiology, Uppsala University, Uppsala, Sweden.

³Laboratoire de Biochimie Métabolique et Osseuse, Université Libre de Bruxelles, Brussels, Belgium

⁴Mycology & Aerobiology, Sciensano, Brussels, Belgium

Correspondence:

Alain Van Muylem, Chest Department,

CUB Erasme, Université Libre de Bruxelles

808 Route de Lennik, 1070 Brussels - Belgium

E-mail: avmuylem@ulb.ac.be

References

1. Drake LY, Kita H. IL-33: biological properties, functions, and roles in airway disease. *Immunol Rev.* 2017;278(1):173-84.
2. Prefontaine D, Nadigel J, Chouiali F, Audusseau S, Semlali A, Chakir J, *et al.* Increased IL-33 expression by epithelial cells in bronchial asthma. *J Allergy Clin Immunol.* 2010;125(3):752-4.
3. Mitchell PD, Salter BM, Oliveria JP, El-Gammal A, Tworek D, Smith SG, *et al.* IL-33 and Its Receptor ST2 after Inhaled Allergen Challenge in Allergic Asthmatics. *Int Arch Allergy Immunol.* 2018;176(2):133-142.
4. Kamekura R, Kojima T, Takano K, Go M, Sawada N, Himi T. The role of IL-33 and its receptor ST2 in human nasal epithelium with allergic rhinitis. *Clin Exp Allergy.* 2012;42(2):218-28.
5. Guo Z, Wu J, Zhao J, Liu F, Chen Y, Bi L, *et al.* IL-33 promotes airway remodeling and is a marker of asthma disease severity. *J Asthma.* 2014;51(8):863-9.

6. Fux M, Pecaric-Petkovic T, Odermatt A, Hausmann OV, Lorentz A, Bischoff SC, *et al.* IL-33 is a mediator rather than a trigger of the acute allergic response in humans. *Allergy*. 2014;69(2):216-22.
7. Cayrol C, Duval A, Schmitt P, Roga S, Camus M, Stella A, *et al.* Environmental allergens induce allergic inflammation through proteolytic maturation of IL-33. *Nat Immunol*. 2018;19(4):375-85.
8. Braunstahl GJ, Fokkens WJ, Overbeek SE, KleinJan A, Hoogsteden HC, Prins JB. Mucosal and systemic inflammatory changes in allergic rhinitis and asthma: a comparison between upper and lower airways. *Clin Exp Allergy*. 2003;33(5):579-87.
9. Berraïes A, Hamdi B, Ammar J, Hamzaoui K, Hamzaoui A. Increased expression of thymic stromal lymphopoietin in induced sputum from asthmatic children. *Immunol Lett*. 2016;178:85-91.
10. Gökkaya M, Damialis A, Nussbaumer T, *et al.* Defining biomarkers to predict symptoms in subjects with and without allergy under natural pollen exposure. *J Allergy Clin Immunol*. 2020;S0091-6749(20)30419-X.
11. Data available on request due to privacy/ethical restrictions

MATERIALS AND METHODS

Patients

Adult patients suffering from seasonal allergic rhinitis (n=27) without asthma symptoms or seasonal intermittent or moderate-to-mild persistent allergic asthma (n=23), sensitized to birch and/or grass pollens, as well as atopic asymptomatic (n=9) and healthy volunteers (n=17), were recruited from the outpatient allergy and asthma clinic in CUB-Erasme University Hospital Brussels, Belgium. Allergic rhinitis and asthma were defined according to the standard criteria: ARIA and GINA guidelines, respectively (1,2).

At the time of inclusion, patients were non-smokers, with no recent history of upper airway infection. Asthma treatment was halted for one week with the exception of on-demand inhaled short-acting beta-2 agonists that were withheld for at least 6 hours before the induced sputum procedure. Similarly, treatment for allergic rhinitis was stopped one week before the procedure. Atopic status was evaluated by skin prick testing and serum specific IgE towards common inhalant allergens.

Healthy volunteers were recruited among subjects with no symptoms of rhinitis or asthma and exhibiting normal lung function and testing negative to common aeroallergens. Atopic asymptomatic subjects had no symptoms of rhinitis or asthma, exhibited normal lung function and tested positive to one or several common aeroallergens. The study was approved by the local ethics committee of CUB Erasme University Hospital Brussels, Belgium (Ethics Committee Number P2014/088). All of the involved patients signed consent forms.

Study design

The study was designed as a prospective open study and carried out in the Chest Department of the Erasme University Hospital, Brussels, Belgium and the Laboratoire de Biochimie Métabolique et Osseuse, Université Libre de Bruxelles, Belgium.

FENO levels, spirometry parameters, and sputum induction were performed twice, once out of the pollen season and once during the pollen season, for allergic rhinitis and asthma patients, and once for healthy controls.

Fractional exhaled nitric oxide

FENO was measured prior to any forced expiratory manoeuvres using a daily calibrated LR 2000 chemiluminescence analyser (Logan Research LTD, Rochester, UK), with the online measurement of a single exhalation at flow rate of 50ml/s (ATS/ERS standard)(3). Exhaled NO levels were read at the plateau corresponding to 70-80% of the CO₂ curve. Absolute FENO values were expressed in parts per billion (ppb).

Lung function

Lung function was measured using a Zan 300 spirometer (Zan®, Oberthulba, Germany) following standard guidelines (4). Forced expiratory volume in 1 second (FEV₁), was expressed as a percentage of the predicted value (% pred).

Sputum induction and processing

Sputum was induced by inhaling 50 ml hypertonic saline (NaCl 5%) from an ultrasonic nebuliser (Ultra-Neb 2000, Devilbiss) with an output set at 0.93 ml/min, according to ERS recommendations(5).

Each subject inhaled the aerosol for four consecutive periods of 5 min, for a total duration of 20 min.

The entire amount of resulting sputum was collected in a plastic container, weighed and homogenised by adding three volumes of phosphate-buffered saline (PBS), vortexed for 30 sec and centrifuged at 800 g for 10 min at 4°C. Supernatant was separated from cell pellet. We then added DTT (dithiotreitol) to the cells, which were agitated for 20 min. These cells were once again washed with PBS and re-suspended in 1 ml. Squamous cells, total cell counts and cell viability, checked by trypan blue exclusion, were performed using a manual haemocytometer. When squamous cells were >70%, the sample was deemed inappropriate.

Sputum cell count

The differential cell count was performed on cytopins stained with Diff-Quick after counting 400 cells.

RNA extraction and cDNA synthesis

Total RNA isolation from cells pellets were extracted using AURUM™ total RNA fatty acid and tissue kit (BioRad, Hercules, CA, USA) according to manufacturer's protocole. The quantity was measured using Nanodrop spectrophotometer (NanoDrop Technologies, Inc, Wilmington, DE, USA). The quality was determined by the 260/280 ratio ranged from 1.8 to 2.0. Purified RNA, was reverse-transcribed 100 ng total RNA using the iScript™ cDNA Synthesis kit (BioRad, Hercules, CA). Quantitative real time was performed on a CFX 96 Real-Time System C1000 Thermal Cycler (BioRad).

Real Time quantitative PCR

Quantitative real-time PCR was applied on a CFX 96 Real-Time System C1000 Thermal Cycler (BioRad). All reaction were made in duplicate, the reaction mixed consisted in 2 µL of each cDNA, forward and reverse primers at 300 nM final concentration, 5,6 microL DEPC water and 10 µL IQ™ SYBR™ Green Supermix (Bio-Rad). The cycling condition were: 3 min at 95°C followed of 40 cycles at 95°C for 15 sec, 60°C for 30 sec, finally 65°C for 5 sec and 95°C for 5 sec. The primers sequences were designed with Primer 3.0 Software from GenBank and synthetized by (Eurogentec , Seraing, Belgium).

The sequences were for hIL 33 forward : TGGTAAAGAAACGGTCAACATTC, reverse: GCAGCTCTAGTGTTTGAGCC

and hGAPDH forward : CAGCCTCAAGATCATCAGCA, reverse: GGTCGTCATGAGTCCTTCCA.

Relative quantification values were calculated by the $2^{-\Delta\Delta CT}$ method. Levels of respective transcripts were normalized to Glyceraldehyde-3- phosphate deshydrogenase (GAPDH) transcript level. ΔCT was calculated by the difference between the cycle threshold (CT) of the target amplicon and (CT) for housekeeping gene (GAPDH). $\Delta\Delta CT$ was obtained by subtraction of values of ΔCT for cDNA from sputum cells of healthy subject from ΔCT for cDNA of each asthmatic or rhinitis samples. The relative quantity (QR) of each samples was calculated using $2^{-\Delta\Delta CT}$ formula.

Enzyme Linked Immunosorbent Assay (ELISA)

The levels of human IL-33 were measured in supernatants of induced sputum, in accordance with the manufacturer's instructions; using commercial kit Human HS Cytokine B premixed Mag Luminex (R&D Systems, Inc., Minneapolis, MN, USA). The detection limits were 0.187-1.65 pg/ml.

The amount of human IL-5 in supernatants of induced sputum was measured using commercial Magnetic Luminex Performance assay kit (R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacture's instruction. The BioPlex® MAGPIX™ (BioRad) was used to read plate. The lower limit of detection for this cytokine was 0,12 pg/mL.

Airborne pollen monitoring

Exposure to airborne pollen was monitored by the Belgian Aerobiological Surveillance Network, which is managed by Sciensano (Brussels, Belgium). Air sampling was performed with a Hirst-type seven day volumetric spore trap (Burkard Manufacturing Co., Rickmansworth, UK), located in Brussels (latitude 50.82, longitude 4.38) at 16 m height above the ground level (6). Pollen from birch trees (*Betula* spp.), grasses (Poaceae) and mugwort (*Artemisia* spp.) were identified and counted by light microscopy (two longitudinal slide lines reading). Mean daily pollen concentration was calculated and expressed as pollen grains per cubic meter of air.

Statistical analysis

Differences in sex ratio between studied populations were analyzed by an exact Fisher test. Difference between studied populations in term of age, FEV1 (in % predicted) and FENO (log-transformed) were analyzed by a one way analysis of variance followed, if necessary, by a Tuckey post-hoc procedure. Differences in term of sputum eosinophils, ELISA IL-33, ELISA IL-5, and qPCR IL-33 were analyzed by non-parametric Kruskal-Wallis test followed, if necessary, by a Dunn post-hoc procedure. Non-parametric Wilcoxon paired tests for sputum

eosinophils, ELISA IL-33, and ELISA IL-5, and paired t-test for FENO were used to study the differences between non-exposure and exposure to allergen.

The R software (release 3.5.2) was used for the analyses. Statistical significance was defined as a p-value of less than 0.05 (two-tailed) (7).

References

1. Brozek JL, Bousquet J, Agache I, Agarwal A, Bachert C, Bosnic-Anticevich S, et al. Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines-2016 revision. J Allergy Clin Immunol. 2017;140(4):950-8.
2. Global Initiative for Asthma [Available from: www.ginasthma.org.
3. American Thoracic S, European Respiratory S. ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. Am J Respir Crit Care Med. 2005;171(8):912-30.
4. Quanjer PH, Tammeling GJ, Cotes JE, Pedersen OF, Peslin R, Yernault JC. Lung volumes and forced ventilatory flows. Eur Respir J. 1993;6 Suppl 16:5-40.
5. Efthimiadis A, Spanevello A, Hamid Q, Kelly MM, Linden M, Louis R, et al. Methods of sputum processing for cell counts, immunocytochemistry and in situ hybridisation. Eur Respir J Suppl. 2002;37:19s-23s.
6. Buters JTM, Antunes C, Galveias A, Bergmann KC, Thibaudon M, Galan C, et al. Pollen and spore monitoring in the world. Clin Transl Allergy. 2018;8:9.
7. team R. R Core Team R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2018 [Available from: <https://www.R-project.org/>].