






Ozge Soyer  <https://orcid.org/0000-0002-7444-251X>
 Umit Murat Sahiner  <https://orcid.org/0000-0003-0088-913X>
 Bulent Enis Sekerel  <https://orcid.org/0000-0001-7402-6850>

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SUPPORTING INFORMATION

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

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Epidemic thunderstorm asthma susceptibility from sensitization to ryegrass (*Lolium perenne*) pollen and major allergen Lol p 5

To the Editor,

Thunderstorm asthma risk in geographic regions with temperate grasses is strongly correlated with the trifecta of ryegrass pollen (RGP) sensitization (serum RGP-specific IgE), seasonal allergic rhinitis (SAR), and exposure to a thunderstorm during the pollen season.^{1,2} Perennial ryegrass (*Lolium perenne*) is a wind-pollinated pasture grass prevalent in southeastern Australia, North America, and Southern Europe. Importantly, RGP-sensitized patients with SAR even without a previous doctor diagnosis of asthma may, in the presence of the trifecta, experience bronchoconstriction, known as epidemic thunderstorm asthma (ETSA).^{1,3,4} In SAR, nasal and ocular symptoms and signs are typically elicited when intact RGP grains (≥ 30 microns) lodge in the upper airways. In contrast, it is suggested that ETSA is triggered when thunderstorm moisture ruptures RGP grains through osmotic shock to release respirable (< 3 micron) allergen-impregnated starch granules which then trigger bronchoconstriction.⁵ Immunologically, the two most prevalent major allergens of *Lolium perenne* are Lol p 1 (30 kDa) and Lol p 5 (29-31 kDa; formerly called Lol p IX), with each eliciting serum IgE reactivity in $> 90\%$ of RGP-sensitized individuals.⁶ Lol p 1 is initially presented in the cytosol of pollen grains, from which it is subsequently secreted to coat their surface. It is then readily leached in soluble form from grains lodged in the upper airways, triggering SAR. In contrast, Lol p

5 resides in the starch granules (amyloplasts) that are released following pollen grain rupture and respired into the lower airways during thunderstorms.⁷ Similarly, levels of airborne group 5 allergens for *Phleum pratense* (Phl p 5) in respirable-sized particles are positively correlated to relative humidity.⁸ Furthermore, while the majority of individuals sensitized to RGP might be expected to also be sensitized to both Lol p 1 and Lol p 5, the degree of sensitization to each major allergen may vary between individuals. The late Bruce Knox (Professor of Botany, University of Melbourne, Australia) hypothesized that RGP starch granules associated with Lol p 5 might be responsible for triggering an epidemic of thunderstorm asthma.⁹

To test the Knox hypothesis, and to determine whether levels of sensitization to RGP generally, and Lol p 5 specifically, have diagnostic utility for predicting risk of ETSA, we quantitated the relevant serum specific (sp) IgE using enzyme-linked immunosorbent assays (ELISA). Serum for ELISA was obtained from the blood of 60 patients who presented to the Alfred Hospital Emergency Department due to the catastrophic ETSA event of 21 November 2016.² For comparison, the same analysis was performed on serum drawn from 19 control individuals recruited from the Asthma & Allergy Clinic with symptoms of SAR, and who were present in Melbourne and outdoors on 21 November 2016, but who did not experience ETSA. The ETSA group had an age distribution similar to the control group but

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TABLE 1 Patient characteristics

	Thunderstorm asthma patients (n = 60)	Nonthunderstorm asthma controls (n = 19)
Age, year (mean ± SD)	39.8 ± 15.4	36.9 ± 12.0
Female	24 (40%)	13 (68%)
Springtime allergic rhinitis symptoms	60 (100%)	19 (100%)
Current asthma before thunderstorm	23 (38%)	8 (37%)
Total IgE, kU/L (median, IQR)	170 (93-571)	213 (88-475)
Ryegrass pollen-specific IgE, kU/L (median, IQR)	51.5 (25.6-100.0)	16.7 (4.1-49.5)
Lol p 1-specific IgE, µg/mL (median, IQR)	1.28 (0.55-3.45)	1.15 (0.29-2.09)
Lol p 5-specific IgE, µg/mL (median, IQR)	2.61 (1.37-4.05)	1.70 (0.17-2.64)

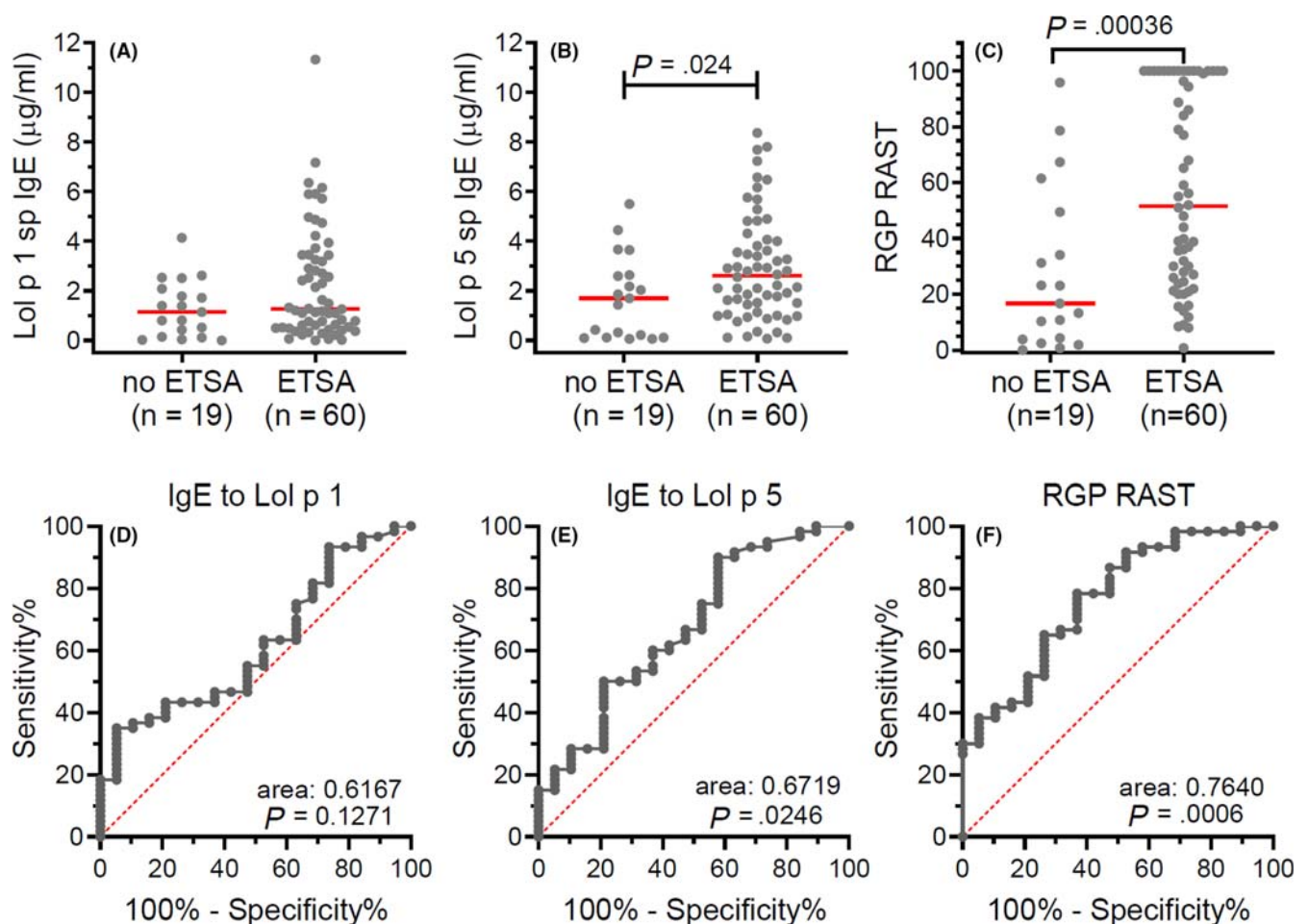


FIGURE 1 A-C, Specific IgE to Lol p 1, Lol p 5, and RGP, respectively, in patients with and without ETSA. Individual measurements are depicted as gray dots with red bars representing medians. Statistics, nonparametric Mann-Whitney *U* test. D-F, Receiver operator characteristics of specific IgE to Lol p 1, Lol p 5, and ryegrass pollen, respectively, for ETSA. Statistics, Wilson/Brown method to test whether the confidence level of the outcome distribution is greater than 95%

a greater proportion of male patients (Table 1). Detailed information about this study is available in this article's online repository.

For analysis of sp IgE, recombinant Lol p 1 (rLol p 1) was purchased (MyBiosource, San Diego, CA) and recombinant Lol p 5 (rLol p

5)¹⁰ was produced in-house in the insect cell line Sf21 using a baculovirus system. ETSA patients had a mean Lol p 1 sp IgE concentration similar to control participants, but higher mean RGP sp IgE and Lol p 5 sp IgE (Table 1, Figure 1A-C). Using receiver operator characteristic

(ROC) curve characteristics, both Lol p 5 sp IgE and RGP sp IgE levels distinguished ETSA-affected patients from controls with an area under the curve (AUC) of 0.67 and 0.76, respectively, while Lol p 1 sp IgE had no such diagnostic utility (Figure 1D-F).

Mechanistically, our results support the Knox hypothesis.⁹ We show that susceptibility to ETSA is linked with greater sensitization to Lol p 5 found on the respirable starch granules, but not Lol p 1. These data have important ramifications for clinical practice. The absolute level of sp IgE to RGP, and to a lesser extent to Lol p 5, was features associated with those susceptible to ETSA. Given high rates of ryegrass pollen sensitization in ETSA areas (of up to 40% in Melbourne, Australia),² it is not feasible to implement protective strategies in all sensitized patients. However, our data suggest that the degree of sensitization can contribute to the assessment of absolute risk, to help focus attention on the patient subgroup most suitable for specific preventive measures. The future development of accurate risk prediction tools may benefit from combining immunological markers of risk with clinical risk factors such as concurrent asthma and past history of ETSA. Until Lol p 5 in vitro testing becomes routinely available, it may also be useful to explore the utility of specific IgE to Phl p 5 (as representative of highly cross-reactive group 5 allergens).⁸

Risk stratification is particularly relevant because at least two protective strategies are now indicated for ETSA. Case-control data suggest inhaled corticosteroid use is beneficial,¹¹ while controlled trial results supported by evidence of immunological tolerance indicate RGP allergen immunotherapy protects from ETSA,^{12,13} so both options should be considered in high-risk individuals.

While every patient in the ETSA group was clearly susceptible to ETSA, we could not be completely certain that all individuals in the control group were protected, since allergen exposure during the 2016 thunderstorm epidemic may have varied between different outdoor locations. There is therefore a possibility that ETSA-susceptible individuals may have been clinically misclassified as "protected." Such classification error, if present, would tend to dilute observable between-group differences, so we may have underestimated the discriminatory utility of specific IgE to Lol p 5 and RGP. We acknowledge the sample size in this study was small, and the scientific community should prepare for a broader collection of clinical samples in the event of future ETSA events, in order to explore these and other immunological mechanisms.

To conclude, the utility of RGP and Lol p 5 sp IgE measurements should be explored further as potential indicators of RGP-related ETSA risk. In particular, the development of risk prediction tools utilizing these immunological factors in combination with clinical features may enable more accurate risk prediction for RGP-related ETSA among SAR patients, in order to offer appropriate protective strategies including allergen-specific immunotherapy.

CONFLICT OF INTEREST

MH has received grants-in-aid, speaker fees, and fees for serving on the advisory boards of GlaxoSmithKline, AstraZeneca, Novartis, Teva, Sanofi, and Seqirus, all paid to his institutional employer Alfred Health. ROH is a minority shareholder of two early-stage

biotechnology companies—Aravax Pty Ltd and Paranta Bio Pty Ltd (in respect of both of which she is a named inventor of the IP assets)—and as such may benefit in the future if the respective experimental medicines are approved for use. All other authors declare no conflicts of interest.

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Mark Hew^{1,2} 

Joy Lee^{1,2}

Nirupama Varese^{3,4}

Pei M. Aui⁴

Craig I. McKenzie⁴

Bruce D. Wines^{4,5,6}

Heather Aumann⁷

Jennifer M. Rolland^{3,4}

Phillip Mark Hogarth^{4,5,6}

Menno C. van Zelm^{3,4} 

Robyn E. O'Hehir^{1,3,4}

¹Allergy, Asthma & Clinical Immunology, Alfred Health, Melbourne, VIC, Australia

²School of Public Health and Preventive Medicine, Monash University, Melbourne, VIC, Australia

³Department of Allergy, Immunology and Respiratory Medicine, Central Clinical School, Monash University and Alfred Health, Melbourne, VIC, Australia

⁴Department of Immunology and Pathology, Central Clinical School, Monash University, Melbourne, VIC, Australia

⁵Immune Therapies Group, Burnet Institute, Melbourne, VIC, Australia

⁶Department of Clinical Pathology, University of Melbourne, Parkville, VIC, Australia

⁷Department of Haematology, Alfred Health, Melbourne, VIC, Australia

Correspondence

Mark Hew, Respiratory Medicine, The Alfred Hospital, 55 Commercial Road, Melbourne, Victoria 3183, Australia.

Email: m.hew@alfred.org.au

ORCID

Mark Hew  <https://orcid.org/0000-0002-7498-0000>

Menno C. van Zelm  <https://orcid.org/0000-0003-4161-1919>

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SUPPORTING INFORMATION

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

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The effect of air pollutants on airway innate immune cells in patients with asthma

To the Editor,

Air pollution is becoming a serious health risk to patients with chronic diseases, especially asthma. Particulate matter (PM), diesel exhaust particles (DEP), ozone (O₃), and other chemicals and pollutants have been shown to have a detrimental effect on respiratory function.¹ However, the mechanisms through which each air pollutant aggravates asthma symptoms are not clearly understood.

Innate lymphoid cells (ILCs) have gained attention due to their potent role in regulating allergic disorders by producing diverse cytokines in response to different stimuli.² In a previous study, we demonstrated that ILCs can regulate asthma phenotypes by interacting with other innate immune cells in human asthmatics.³ In the current study, we aimed to determine (a) how ILCs and macrophages respond to various air pollutants and (b) how these cells affect asthma symptoms.

A total of 118 subjects (50 healthy subjects, 52 mild asthma patients, and 16 severe asthma patients) were recruited (Table S1), and innate immune cells including ILCs and macrophages were analyzed in the induced sputum (Figure S1). Initially, ILCs defined as CD45⁺, lineage-negative (Lin⁻) cells that express CD127. To subdivide ILC populations, ST2, c-Kit, and NKp44 makers were used. CD45⁺CD68⁺ cells were considered as macrophages, and M1, M2, and alveolar macrophages were further identified by CD11c and CD206 expression

(Figure S1A and B). First, to verify the impact of air pollutants on asthma, we analyzed the relationship between the concentrations of air pollutants [particulate matter with a diameter ≤ 10 μm (PM₁₀), particulate matter with a diameter ≤ 2.5 μm (PM_{2.5}), Ozone (O₃), nitrogen dioxide (NO₂), carbon monoxide (CO), and sulfur dioxide (SO₂)] measured in the residential area of the patients on the day of getting sputum, asthma symptom scores, and lung function measurements from participants, including healthy controls (Table S2). Contrary to general expectations, most air pollutants had little or no effect on the asthma index. Among the various air pollutants, only the concentration of PM₁₀ in their residential area correlated with the asthma control questionnaire (ACQ) and the asthma control test (ACT) scores. The ratio of forced expiratory volume in 1 second (FEV₁) to forced vital capacity (FVC), a critical indicator of asthma, was not affected by air pollutants except for NO₂ (Table S2). Based on the hypothesis that air pollutants might affect asthmatics more strongly than healthy controls, we reanalyzed the effect of air pollutants specifically in patients with asthma (Figure 1A-F). In patients with asthma as well, PM₁₀ in their residential area had the greatest impact on ACQ and ACT.

Next, we examined the correlation between the concentration of air pollutants and the frequency of ILCs and macrophages (Table S3). Linear regression analysis showed that only the frequency of ILC2s