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DR. CAROLINE LODGE (Orcid ID : 0000-0002-2342-3888)

DR. ADRIAN LOWE (Orcid ID : 0000-0002-4691-8162)

DR. FAHAD MOHAMMED ALDAKHEEL (Orcid ID : 0000-0001-8047-8650)

DR. BIRCAN ERBAS (Orcid ID : 0000-0001-9597-418X)

DR. SHYAMALI C DHARMAGE (Orcid ID : 0000-0001-6063-1937)

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N. Sabrina Idrose, MSc[a], Rachel C.A. Tham, PhD[b], Caroline J Lodge, PhD[a], Adrian J Lowe, PhD[a], Dinh Bui, PhD[a], Jennifer L Perret, PhD[a], Don Vicendese, PhD[a,c], Edward J Newbigin, PhD[d], Mimi L.K. Tang, MD, PhD[e,f], Fahad M Aldakheel, PhD[g], Nilakshi T Waidyatillake, MD, PhD[a], Jo A Douglass, MD[h,i], Michael J Abramson, MD, PhD[j], E. Haydn Walters, DM[a,k], *Bircan Erbas, PhD[l], *Shyamali C Dharmage, MD, PhD[a]

*Equally contributed

a. Allergy and Lung Health Unit, Melbourne School of Population and Global Health, The University of Melbourne, 207 Bouverie St, Carlton, Victoria, 3053, Australia.
b. Mary MacKillop Institute for Health Research, Australian Catholic University, 215 Spring St, Melbourne, Victoria, 3000, Australia.
c. Department of Mathematics and Statistics, La Trobe University, Bundoora, 3086, Victoria, Australia.

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d. School of BioSciences, The University of Melbourne, Parkville, Victoria, 3052, Australia.
e. Allergy and Immunology, Murdoch Children's Research Institute, Royal Children's Hospital, Melbourne, Victoria, 3052, Australia.
f. Department of Pediatrics, University of Melbourne, Melbourne, VIC, Australia.
g. Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia.
h. Department of Clinical Immunology and Allergy, Royal Melbourne Hospital, Parkville, 3050, Victoria, Australia.
i. Department of Medicine, University of Melbourne, Victoria, 3052, Australia.
j. School of Public Health & Preventive Medicine, Monash University, Melbourne, 3004, Australia.
k. School of Medicine, University of Tasmania, Hobart, Tasmania, 7000, Australia.
l. School of Psychology and Public Health, La Trobe University, Bundoora, 3086, Victoria, Australia.

Corresponding Author: Shyamali Dharmage
Centre of Epidemiology and Biostatistics,
Melbourne School of Population and Global Health,
The University of Melbourne,
207 Bouverie St, Carlton, VIC 3052
Tel: +61 3 83440737
Fax : +61 3 93495815
Email: s.dharmage@unimelb.edu.au

Authors' contributions: Sabrina Idrose: Investigation, Writing- Original Draft. **Rachel Tham:** Investigation, Writing- Review & Editing, Supervision. **Caroline Lodge:** Writing- Review & Editing. **Adrian Lowe:** Writing- Review & Editing. **Dinh Bui:** Writing- Review & Editing. **Jennifer Perret:** Writing- Review & Editing. **Don Vicendese:** Writing- Review & Editing. **Edward Newbigin:** Writing- Review & Editing. **Mimi Tang:** Writing- Review & Editing. **Fahad Aldakheel:** Investigation, Writing- Review & Editing. **Nilakshi Waidyatillake:** Writing- Review & Editing. **Jo Douglass:** Writing- Review & Editing. **Michael Abramson:** Writing- Review &

Editing. **Haydn Walters:** Writing- Review & Editing. **Bircan Erbas:** Conceptualization, Investigation, Writing- Review & Editing, Supervision. **Shyamali Dharmage:** Conceptualization, Investigation, Writing- Review & Editing, Supervision.

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Abstract

Background: The association between grass pollen exposure and early markers of asthma exacerbations such as lung function changes and increase in airway inflammation is limited. We investigated the associations between short-term grass pollen exposure and lung function and airway inflammation in a community-based sample, and whether any such associations were modified by current asthma, current hay fever, pollen sensitization, age and other environmental factors.

Methods: Cross-sectional and short-term analyses of data from the Melbourne Atopy Cohort Study (MACS) participants (n=936). Lung function was assessed using spirometry. Airway inflammation was assessed by fractional exhaled nitric oxide (FeNO), and exhaled breath condensate pH and nitrogen oxides (NOx). Daily pollen counts were collected using a volumetric spore trap. The associations were examined by linear regression.

Results: Higher ambient levels of grass pollen 2 days before (lag 2) were associated with lower mid-forced expiratory flow ($FEF_{25-75\%}$) and FEV_1/FVC ratio (Coef. [95% CI] = -119 [-226, -11] mL/s and -1.0 [-3.0, -0.03] %, respectively) and also 3 days before (lag 3). Increased levels of grass pollen a day before (lag 1) was associated with increased FeNO (4.35 [-0.1, 8.7] ppb) and also at lag 2. Adverse associations between pollen and multiple outcomes were greater in adults with current asthma, hay fever and pollen sensitization.

Conclusion: Grass pollen exposure was associated with eosinophilic airway inflammation 1-2 days after exposure and airway obstruction 2-3 days after exposure. Adults and individuals with asthma, hay fever and pollen sensitization may be at higher risk.

Keywords: Airway inflammation, environmental health, grass, lung function, pollen

Abbreviations: ATS/ERS: American Thoracic Society/European Respiratory Society; BDR: bronchodilator response; CI: confidence interval; EBC: exhaled breath condensate; ED: emergency department; $FEF_{25-75\%}$: mid-forced expiratory flow; FeNO: fractional exhaled nitric oxide; FEV_1: forced expiratory volume in the first second; FVC: forced vital capacity; IgE: Immunoglobulin E; ISAAC: International Study of Asthma and Allergies in Childhood; MACS: Melbourne Atopy Cohort Study; MAPCAH: Melbourne Air Pollen Children and Adolescent Health; NOx: nitrogen oxide; RV: residual volume; SD: standard deviation; SPT: skin-prick test; Th2: T helper 2 lymphocytes

Introduction

Asthma is currently estimated to affect 339 million people worldwide and has become a global public health problem over the last few decades[1]. It affects people of all ages and one of the top contributors to preventable hospitalizations due to acute exacerbations[2]. Identification of acute asthma triggers is a priority to inform preventive strategies to minimize individual episodes as well as societal healthcare costs.

Exposure to temperate grass pollen is being recognized as an increasingly important trigger for acute asthma including thunderstorm asthma[3-5]. Temperate grass pollen contains several allergens, particularly Group 1 and 5, which have considerable homology across grass species[6, 7]. While whole grass pollen grains (10-100μm) usually get trapped in the upper airways, rupture of grass pollen grains during thunderstorms produces hundreds of tiny allergenic starch granules (0.1-5μm) that can penetrate deeper into the lower airways[8]. Furthermore, with changing climatic conditions, the intensity and duration of the grass pollenating period is increasing in many parts of the world[9], increasing the potential impact of grass pollen exposure on health.

While exposure to grass pollen is increasingly recognized as a trigger of acute asthma attacks leading to hospital attendance, this is only the "tip of the iceberg" as there is likely to be a much higher burden of more moderate asthma exacerbations that do not lead to hospitalization within the general community[10]. Despite this, community-based studies investigating the role of pollen on early markers of asthma exacerbations such as lung function changes and increases in airway inflammation have been inconsistent and limited in terms of methodology.

Most studies[11-13] have included selected samples with small number of participants, limiting the study power and generalizability. Of the very few population-based studies with adequate sample sizes, key confounding variables such as temperature and humidity were not considered[14, 15]. More importantly, lack of investigation into factors that may modify these associations such as current asthma, pollen sensitization, hay fever and age has made it difficult to establish high-risk groups who need to be targeted for monitoring or interventions during grass pollen seasons. Furthermore, it has been suggested that the impact of grass pollen exposure is higher in the presence of fungi[16,

[17], rainfall[18], air pollutants[19], higher temperature[20] and lower humidity[21] but these important potential effect modifiers have not been investigated.

Given the above gaps in knowledge, we investigated the associations between grass pollen exposure on the day of clinical testing and the preceding three days and lung function and airway inflammation in a general population cohort at overall at potentially greater risk for asthma and allergies. We examined for susceptible groups and periods by exploring effect modifications by current asthma, pollen sensitization, current hay fever, age and other environmental factors.

Methods

Study design and population

The study sample consisted of participants of the Melbourne Atopy Cohort Study (MACS)[22]. The original cohort consisted of 620 'high risk' babies born between 24 March 1990 and 1 November 1994 in Melbourne, Australia. Each had at least one first-degree relative with a history of allergic diseases i.e. eczema, asthma, hay fever or severe food allergy. The recruitment process and study methodology has been described in detail elsewhere[22, 23].

The present work is based on cross-sectional data from the 18-year follow-up visit. During this follow-up, parents and siblings were also invited to participate. However, only the participants who attended the laboratory between September 2009 and December 2011 were included in the analysis as daily outdoor grass pollen counts were only available during this period. These participants underwent a single episode of clinical testing for lung function (spirometry), airway inflammation (exhaled NO and exhaled breath condensate), and skin-prick tests (SPTs) during the study period. These one-time visits were random in terms of when the individuals attended the clinic relative to the phase of the pollen season. Environmental pollen data were obtained for the day of lab testing (lag 0) and the three days prior to attendance (designated lag day 1, lag day 2 and lag day 3).

Ethics approval was granted by The University of Melbourne and Royal Children's Hospital Human Research Ethics Committees. Participants also provided written informed consent.

Environmental data collection

Daily ambient outdoor grass pollen and fungi levels were collected between September 2009 and December 2011 for the Melbourne Air Pollen Children and Adolescent Health (MAPCAH) study[24]. These aeroallergens were collected using a volumetric spore trap (Burkard, UK) located on the rooftop of the Earth Sciences building at The University of Melbourne. For this study, we analyzed grass pollen, *Cladosporium, Leptosphaeria, Alternaria* and smuts as they were found to be most abundant in Melbourne[25, 26]. The collection method is detailed in the Supplementary Material.

Air pollutants including the daily maximum 4-hour average ozone (O_3), the daily maximum 1-hour average nitrogen oxide (NO_2) and the daily 24-hour average concentrations of particulate matter up to 2.5μm in diameter ($PM_{2.5}$) and up to 10μm in diameter (PM_{10}) during the study period were obtained from the Victorian Environmental Protection Authority. The daily maximum temperatures, rainfall and relative humidity data were obtained from The Bureau of Meteorology.

Clinical data collection

Lung function was measured following ATS/ERS guidelines[27] using the EasyOne Spirometer (ndd Medical Technologies Inc., Andover, MA). Associations with the following pre-bronchodilator (pre-BD) spirometry parameters were assessed: forced expiratory volume in the first second (FEV_1), forced vital capacity (FVC), mid-forced expiratory flow ($FEF_{25-75\%}$) and FEV_1/FVC ratio. Bronchodilator response (BDR) after 400μg inhaled salbutamol was measured as a continuous and dichotomous variable. "Absolute BDR" was defined as the difference between post- and pre-bronchodilator FEV_1 values, while a "positive BDR" response was defined as having $\geq 12\%$ change and ≥ 200ml of absolute change in FEV_1 from pre-bronchodilator to post-bronchodilator measurements[28, 29]. Pollen sensitization to mixed grass and/or ryegrass (Bayer, Spokane, WA, USA) allergen was determined by a skin-prick test (SPT) on the forearm. The measurement details are provided in the Supplementary Material.

Fractional exhaled nitric oxide (FeNO) was measured following ATS/ERS guidelines[30] using the HypAir™ FeNO machine (Medisoft, Sorinnes, Belgium). Exhaled breath condensate (EBC) was collected only in probands (but not relatives) and assayed according to the ATS/ERS guidelines[31,

[32] for acidity (pH) and nitrogen oxides (NOx) using a glass-condensing chamber in wet ice. The methodology of EBC collection is detailed elsewhere[33].

Clinical definitions

Participants were considered to be pollen-sensitized if they had a wheal diameter of at least 3mm greater than saline for any of the grasses tested (i.e. mixed grass and/or ryegrass). Current asthma and hay fever status were identified using a valid ISAAC questionnaire [34]. The participants were considered to have current asthma if they had a history of doctor-diagnosed asthma and one or more of the following in the 12 months prior to the follow-up: (a) wheeze; (b) at least one episode of asthma; (c) use of asthma medication. This definition of current asthma is in line with previous publications from this cohort and did not include BDR, since this might not be a reliable marker for asthma[35]. Current hay fever was defined as having at least one episode of hay fever and/or the use of medication for the symptoms in the 12 months prior to the follow-up. Age was stratified into two groups: (a) children and adolescents ($<$ 18 years old) and (b) adults ($\geq$ 18 years old).

Statistical analysis

The associations between grass pollen exposure on the same day of clinical testing (lag 0) and lagged grass pollen exposure (lag day 1 up to lag day 3 and cumulative exposure 3 days prior) and lung function outcomes (i.e. FEV_1, $FEF_{25\text{-}75\%}$, FEV_1/FVC ratio, absolute BDR); and airway inflammation outcomes (i.e. FeNO and EBC pH) were assessed using generalized linear models. Positive BDR was assessed using generalized estimating equations. EBC NOx was found to be strongly rightly skewed, so it was stratified into none (below level of detection), low ($\geq$ 2.5 to 20 μmol) or high ($>$ 20μmol)[36] and analyzed using ordinal logistic regression (OLR). All models were accounted for familial clustering. In addition, we conducted a sensitivity analysis investigating grass pollen exposure at cumulative lag 7 (average exposure over the prior 7 days).

The multivariable models were adjusted for age, sex, height, maximum temperature, relative humidity, ozone and season of testing as *a priori* confounders[37-41]. Models assessing EBC pH and EBC NOx were also adjusted for storage time to account for possible sample degradation. Potential effect modifications by current asthma (irrespective of hay fever and pollen sensitization status), current hay fever (irrespective of asthma and pollen sensitization status), pollen sensitization (irrespective of hay fever and asthma status), age, air pollutants, ambient fungal levels, temperature, humidity and rainfall were explored separately using Wald's test. We also conducted an exploratory analyses of the relevant associations by the combined phenotypes of current asthma and hay fever (current asthma only, hay fever only or both), pollen sensitization and current hay fever (pollen sensitization only, hay fever only or both), and pollen sensitization and current asthma (pollen sensitization only, current asthma only or both), separately using Wald's test .

The coefficients and odds ratios (OR) were reported as the effect size per increase in 29 grass pollen grains/m^3. Significant associations were defined as $p \leq 0.05$ and p-int ≤ 0.1. Strata-specific associations were only reported if the p-value for interaction term was ≤ 0.1. All statistical analyses were performed using Stata IC 14.2 (StataCorp, College Station, Texas).

Results

Sample characteristics

This study included altogether 936 participants, consisting of 264 probands, plus some parents (n=398) and siblings (n=274). The median (range) age of the study participants was 21 years (6-71). Of the total sample, 45% were males, 30% reported they had current asthma, 13% reported they had current hay fever, and 47% were sensitized to at least one grass pollen. These results are summarized in Table 1. Of those with current asthma, 64.8% were sensitized to at least one grass pollen extract and 19.6% reported current hay fever. The proportion of people with current asthma who attended the clinic each month did not vary (Table S1).

Grass pollen distribution

The 2009 to 2011 study period captured three grass pollen seasons, from September to February, peaking in October to January[42]. There were no distinct variations in the grass pollen seasons between these years. Although a minor thunderstorm asthma event was reported on the 25th of November 2010[4], none of the participants attended the clinic on this day and up to 12 days later.

Grass pollen levels were negatively correlated with relative humidity and nitrogen dioxide levels; and positively correlated with fungal levels (*Cladosporium, Leptosphaeria, Alternaria*, Smuts), maximum temperature, PM_{10} and ozone. The summary statistics and the Spearman's correlations between grass pollen and other environmental factors are presented in Table S2.

Adjusted analyses

The unadjusted (Table S3) and adjusted results (Table 2) were consistent. For adjusted lung function analyses, there were significant inverse associations between grass pollen exposure at lag 2 and lag 3 and $FEF_{25\text{-}75\%}$ and FEV_1/FVC ratio. For adjusted airway inflammation analyses (Table 2), there were significant positive associations between grass pollen exposure at lag 1 and lag 2 and FeNO. The associations at cumulative lag 7 were not significant (data not shown).

Effect modification

Some of the observed associations were modified by current asthma and age (Table 3). Exposure to higher levels of grass pollen at lag 0, lag 1, lag 2 and cumulative lag 3 were associated with an increase in absolute BDR and FeNO in subjects with current asthma (irrespective of hay fever status) but not those without current asthma (p-values for interaction range from 0.004 to 0.06). Furthermore, exposure to higher levels of grass pollen was associated with a significantly lower $FEF_{25\text{-}75\%}$ at lag 2, lag 3 and cumulative lag 3; and FEV_1/FVC ratio at all time points in adults aged 18 and above, but not in children or adolescents (p-values for interaction range from 0.009 to 0.1).

The interactions with current hay fever were not significant but the observed associations in those with current hay fever were stronger compared to those without current hay fever (Table S4). Similar can be observed for pollen sensitization (Table S4). While there were some associations observed between grass pollen and lung function in the presence of little to no fungi, the number of associations seen were consistent with chance given the number of interactions tested in this analysis (Table S5). The interactions between grass pollen and air pollutants ($PM_{2.5}$, PM_{10}, ozone, nitrogen dioxide), temperature, rainfall, and humidity were not significant at all (p-values for interaction ≥ 0.2, data not shown).

Interactions between grass pollen and the combined variable of current asthma and hay fever were significant for $FEF_{25\text{-}75\%}$, FeNO and absolute BDR (range: 0.086 to 0.099, see Table S6). In those with hay fever alone (Table 4), increased grass pollen exposure was associated with decreased $FEF_{25\text{-}75\%}$ at all lag periods except lag 0, increased FeNO at all lag periods except lag 2 and increased absolute BDR at lag 2. In those with hay fever and asthma (Table 4), increased grass pollen exposure was associated with increased absolute BDR at all lag periods. In those with asthma alone (Table 4), increased grass pollen exposure was associated with increased absolute BDR at lag 1, lag 2 and cumulative lag 3.

Interactions between grass pollen and the combined variable of pollen sensitization and current hay fever were significant for FEV_1, FVC and FeNO (range: 0.01 to 0.09, see Table S7). The associations were stronger in pollen-sensitized individuals with hay fever compared to pollen-sensitized individuals without hay fever. In pollen-sensitized individuals with hay fever, increased grass pollen exposure was associated with decreased FEV_1 at lag 3 and higher FeNO at lag 0 and lag 1 (Table 5). The direction of association for FVC was consistent, such that pollen-sensitized individuals with hay fever were observed to have lower FVC at all lags associated with increased grass pollen exposure (Table S8).

Interactions between grass pollen and the combined variable of pollen sensitization and current asthma were significant for FeNO (range: 0.0001 to 0.003, see Table S9). Pollen-sensitized individuals with asthma were observed to have higher FeNO at all lags associated with increased grass pollen exposure (Table S10).

Discussion

This analysis has shown that short-term exposure (up to 3 days) to grass pollen was associated with reduced lung function and increased airway inflammation, particularly for $FEF_{25-75\%}$, FEV_1/FVC ratio and FeNO, but with different lag patterns. These associations were stronger in specific groups of individuals – those with current asthma or hay fever, grass pollen-sensitized individuals and adults. All associations were lagged, which is consistent with grass pollen exposure inducing a late inflammatory response[43]. An inflammatory cascade resulting in the recruitment of immune cells such as eosinophils is suggested to peak approximately 24-72 hours after grass pollen exposure[44] which could be the reason why we did not observe significant associations at cumulative lag 7 days. An alternative explanation for the lack of association found at cumulative lag 7 days is the nature of grass pollen seasons in Melbourne. It consists of short periods of 2-3 days with hot/sunny weather with continental anticyclones, interspersed with westerly cold/wet low-pressure fronts. This intermittent weather coupled with the different variations of grass pollen levels makes it an ideal place for such a natural experiment investigating short-term effects of up to 3 days. Interestingly, we observed a more delayed effect on lung function compared to airway inflammation. Lung function was observed to be lower 2-3 days after grass pollen exposure, but airway inflammation was observed to increase 1-2 days after grass pollen exposure. These findings suggest a temporal association between lung function deficits and airway inflammation.

Our observation that grass pollen exposure was associated with $FEF_{25-75\%}$ but not significantly with FEV_1 may indicate that the greatest impact is on medium-sized to small airways. This is consistent with observations by Marseglia et al.[45] in a population with allergic rhinitis. Reduction in FVC in pollen-sensitized individuals with hay fever is consistent with narrowing of the smallest airways affecting air-trapping and so raising Residual Volume (RV) and impinging on FVC. Unfortunately, we did not have RV data to confirm this. Smaller airways have in aggregate far greater cross-sectional surface areas and airway volumes compared to large airways, but they only contribute to 10% of airway resistance[46]. FEV_1 is less sensitive to smaller airway changes[46] so this could be a reason why past studies[47-49] found no association with lung function when they measured FEV_1 alone. While there have been contradictory views on $FEF_{25-75\%}$ as a reliable marker of small airway obstruction and variability in this measure can occur as a result of its close relationship to FEV_1 and FVC[50], $FEF_{25-75\%}$ has been suggested to be a marker of a more distal airway narrowing[51]. We did find small reductions in FEV_1/FVC ratio which were statistically significant but might not be clinically meaningful. In addition, we also observed a significant increase in FeNO, which would be consistent with eosinophilic airway inflammation[52, 53].

Moreover, we observed that the associations between grass pollen exposure, FeNO and absolute BDR were stronger in people with current asthma compared to those without. This is consistent with asthmatics being more susceptible to reversible airflow limitation during peak grass pollen seasons[12]. In asthma, the lung elasticity is further lost, and exposure to grass pollen would lead to increased inflammation. Additionally, we observed a significant association with $FEF_{25-75\%}$, FeNO and absolute BDR among those with hay fever alone, but only with absolute BDR among those with both asthma and hay fever or asthma alone. This was not what we expected because one would speculate that people with current asthma would have more pulmonary function changes to allergen exposure. Nevertheless, the strongest associations in absolute BDR were observed among those with both asthma and hay fever, followed by those with asthma alone. For those with hay fever alone, the changes in absolute BDR were smaller but significant at lag 2, indicating a degree of sub-clinical asthma making them potentially quite vulnerable to a large surge of atmospheric pollen such as in a thunderstorm.

Indeed, grass pollen-sensitized individuals irrespective of current hay fever and asthma status were observed to have increased airway inflammation with increased grass pollen exposure, but pollen-sensitized individuals with either current hay fever or asthma were shown to have greater associations in terms of both lung function and airway inflammation. There was also a modifying effect of age with adults having stronger associations with increased grass pollen exposure, which is consistent with adults being more affected by thunderstorm asthma[4, 54]. Although the response rate for mothers was slightly higher compared to fathers, this matched the distribution of males to females in the study (42% and 58% respectively) and so is unlikely to have biased the findings. Furthermore, reporting bias was unlikely because we used objective lung function measures to assess the outcomes.

Strengths and limitations

To the best of our knowledge, this is the first large, community-based study to investigate the association between grass pollen levels and objective measures of lung function and airway inflammation. Furthermore, clinical and exposure measurements were carried out using gold standard techniques and validated questionnaires to limit measurement bias. The wide range of data collected from this study also allowed investigation into effect modifiers, which had not been previously documented.

We did not find evidence to support the hypothesis of interactions between exposures to fungi and pollen. Nevertheless, this should be examined in future studies, especially given the isolated findings that we observed in relation to few specific fungi taxa. There was also no evidence of interaction by air pollutants and meteorological variables. This could be due to limited power, lack of individual level data (e.g. proximity of residence to major roads) and the relatively low pollution levels in this study setting. Thunderstorm asthma events occur during extreme weather conditions such as an increase in humidity, a sudden drop in temperature and presence of thunderstorm outflows, which result in the increase of pollen allergen load in the air. Unfortunately, we did not have patient data to assess the effects of these extreme weather conditions.

Several other study limitations also need to be considered. There was potential for spurious findings due to multiple hypothesis testing. Grass pollen counts were measured at a single site and this might not have accurately reflected exposure at an individual level. Nonetheless, measurements were made within approximately 50km of the central business district, where all participants resided. Data such as the relative distance between residence and collection site, time spent outdoors and indoor pollen measurements were also not collected, so these would have limited exposure assessment. However, these misclassifications were likely to be non-differential, would bias towards the null and so unlikely to explain the positive results. Furthermore, the cross-sectional approach of this analysis did not consider daily changes in allergen exposure on lung function and airway inflammation as the participants were assessed only once during the study period. This would have provided stronger evidence for the associations found in this study. Moreover, we had limited data to explore grass pollen at the species level and sensitization to specific grass pollen taxa. This would have provided us with a better understanding on the relationship between grass pollen, lung function and airway inflammation. Lastly, the MACS cohort consisted of individuals with a family history of allergic diseases. While this may limit generalizability, approximately 70% of the Australian population has a family history of allergies[55].

In conclusion, the findings from our study highlight the relationship between short-term grass pollen exposure (within 3 days) and subsequent lung function and airway inflammation changes.

Our data suggest that adults and individuals with current asthma, current hay fever and grass pollen-sensitization are especially vulnerable. These findings are fundamental to inform individual and public health preventive strategies for asthma exacerbation and hospital admissions due to grass pollen exposure, such as a preventive asthma therapy or allergen immunotherapy. In addition, the use of modern technologies such as early warning systems on smartphones and social media sites for impending high pollen days, as well as broader information technology systems (ITS)-based public education to the adverse effects of grass pollen exposure could increasingly play an important preventive role.

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Table 1. Characteristics of MACS participants during the study period.

Variables		All	Probands	Mothers	Fathers	Siblings
N (%)		936	264 (28%)	238 (25%)	160 (17%)	274 (29%)
Median age (range) (years)		21 (65)	18 (5)	49 (20)	52 (30)	16 (35)
Mean height ± SD (cm)		169.9 ± 10.2	172.6 ± 1.5	164.2 ± 6.0	177.7 ± 6.4	167.8 ± 11.8
		n (% of all)	**n (% of subgroup)**	**n (% of subgroup)**	**n (% of subgroup)**	**n (% of subgroup)**
Gender	Male	424 (45%)	127 (48%)	-	160 (100%)	137 (50%)
	Female	512 (55%)	137 (52%)	238 (100%)	-	137 (50%)
Mixed grass sensitisation	Tested	n=895	n=253	n=228	n=154	n=258
	Yes	391 (44%)	105 (42%)	103 (45%)	60 (39%)	122 (47%)
Ryegrass sensitisation	Tested	n=896	n=253	n=227	n=154	n=260
	Yes	410 (46%)	109 (43%)	110 (48%)	66 (43%)	124 (48%)
Pollen sensitisation (Any†)	Tested	n=890	n=252	n=224	n=154	n=258
	Yes	422 (47%)	112 (44%)	111 (50%)	68 (44%)	130 (50%)
Current asthma	Total	n=811	n=229	n=207	n=146	n=229

	Yes	247 (30%)	66 (29%)	74 (36%)	28 (19%)	79 (34%)
Current hay fever	Total	n=815	232	205	141	237
	Yes	108 (13%)	23 (9.9%)	38 (19%)	14 (9.9%)	33 (14%)
Spirometry test		873 (93%)	262 (99%)	218 (92%)	146 (91%)	244 (89%)
FeNO test		797 (85%)	222 (84%)	205 (86%)	139 (87%)	231 (84%)
EBC test		231 (24.6%)	231 (88%)	-	-	-

†Sensitisation to mixed grass and/or ryegrass

Table 2. **Adjusted analysis** of grass pollen and various outcomes on same day, lag 1, lag 2, lag 3 and cumulative 3-day lag.

Outcomes	Grass pollen									
	Lag 0		Lag 1		Lag 2		Lag 3		Lag 0-3	
	Coef. (95% CI)	**p-value**	**Coef. (95% CI)**	**p-value**	**Coef. (95% CI)**	**p-value**	**Coef. (95% CI)**	**p-value**	**Coef. (95% CI)**	**p-value**
FEV_1 (ml)	1.45 (-70, 74)	0.97	-16.0 (-70, 38)	0.56	-29.0 (-81, 21)	0.25	-36.0 (-97, 26)	0.26	-7.3 (-23, 8.7)	0.37
FVC (ml)	0.87 (-65, 67)	0.98	-2.0 (-55, 51)	0.94	6.1 (-48, 60)	0.82	-2.9 (-71, 65)	0.93	0.29 (-16, 17)	0.96
$FEF_{25-75\%}$ (ml/second)	-10.1 (-45, 25)	0.58	-66.4 (-171, 38)	0.21	-119 (-226, -11)*	0.03*	-122.1 (-225, -20)*	0.02*	-26 (-56, 3.8)	0.086
FEV_1/FVC ratio (%)	-0.3 (-2.0, 1.0)	0.45	-0.6 (-1.0, 0.3)	0.21	-1.0 (-3.0, -0.03)*	0.04*	-2.0 (-3.0, -0.3)*	0.02*	-0.2 (-0.4, 0.06)	0.15
Absolute BDR (ml)	-1.2 (-19, 17)	0.90	9.0 (-11, 29)	0.37	16 (-4.6, 36)	0.13	13.1 (-7.3, 34)	0.21	2.7 (-2.9, 8.4)	0.34
FeNO (ppb)	-2.9 (-2.0, 8.1)	0.25	4.35 (-0.1, 8.7)*	0.05*	4.35 (-0.001, 8.7)*	0.05*	1.74 (-2.0, 5.5)	0.39	1.0 (-0.18, 2.2)	0.096

EBC (pH)	0.09 (-0.06, 0.3)	0.17	0.12 (-0.01, 0.3)	0.07	0.15 (0.06, 0.3)	0.04	0.15 (-0.01, 0.3)	0.07	0.03 (-0.0003, 0.07)	0.05
	OR (95% CI)	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**
EBC NOx (μmol)	0.75 (0.31, 1.0)	0.31	0.75 (0.41, 1.3)	0.45	0.89 (0.56, 1.8)	0.72	1.0 (0.56, 1.0)	0.92	1.0 (0.7, 1.0)	0.65
Positive BDR	0.75 (0.56, 1.0)	0.50	1.0 (0.75, 1.0)	0.998	1.0 (0.75, 1.78)	0.27	1.0 (0.75, 1.78)	0.72	1.0 (0.94, 1.0)	0.56

N.B. FEV_1, $FEF_{25-75\%}$, absolute BDR and FeNO associations were adjusted for age, sex, height, relative humidity, temperature, ozone and season of testing. FEV_1/FVC ratio associations were adjusted for relative humidity, temperature, ozone and season of testing. EBC associations were adjusted for storage time in addition to the other confounders. All results are interpreted as per increase in 29 grass pollen grains/m^3. *$p \leq 0.05$.

Table 3. **Significant associations** between grass pollen and different outcomes stratified by current asthma and age.

Grass pollen	Outcomes					
	Absolute BDR (ml)			FeNO (ppb)		
	No current asthma **n=517**	**Current asthma** **n=235**		**No current asthma** **n=517**	**Current asthma** **n=235**	
	Coef. (95% CI)	**Coef. (95% CI)**	**p-int**	**Coef. (95% CI)**	**Coef. (95% CI)**	**p-int**
Lag 0	-16 (-37, 4.6)	50 (6.4, 93)	0.004*	-2.9 (-5.8, 2.9)	8.7 (-0.9, 17.4)	0.04*
Lag 1	-15 (-40, 11)	52 (10, 95)	0.004*	-1.2 (-4.4, 2.0)	8.7 (-0.6, 18.0)	0.048*
Lag 2	-5.5 (-33, 22)	46 (6.1, 86)	0.03*	-1.74 (-4.9, 1.4)	8.4 (-0.3, 17.1)	0.03*
Lag 3	-2.6 (-30, 25)	35 (-11, 81)	0.17*	-1.7 (-4.9, 1.7)	2.6 (-5.5, 10.7)	0.31
Lag 0-3	-2.6 (-9.3, 4.1)	13 (1.3, 24)	0.016*	-0.4 (-1.4, 0.5)	2.0 (-0.4, 4.6)	0.06*
	$FEF_{25\text{-}75\%}$ (ml/second)			FEV_1/FVC ratio (%)		
	< 18 **years old** **n=246**	≥ **18 years old** **n=627**		< 18 **years old** **n=246**	≥ **18 years old** **n=627**	
	Coef. (95% CI)	**Coef. (95% CI)**	**p-int**	**Coef. (95% CI)**	**Coef. (95% CI)**	**p-int**
Lag 0	-30 (-115, 176)	-83 (-202, 36)	0.13	0.6 (-0.3, 1.5)	-0.9 (-1.5, -0.0003)	0.009*
Lag 1	43 (-107, 192)	-104 (-221, 13)	0.07*	0.4 (-0.6, 1.5)	-0.9 (-1.7, -0.1)	0.02*
Lag 2	-7.9 (-181, 165)	-142 (-255, -29)	0.10*	0.09 (-1.2, 1.5)	-1.2 (-1.7, -0.3)	0.04*

Lag 3	-22 (-211, 167)	-168 (-280, -57)	0.10*	-0.1 (-1.5, 1.2)	-1.2 (-2.0, -0.3)	0.08*
Lag 0-3	3.2 (-45, 51)	-36 (-67, -4.9)	0.09*	0.06 (-0.3, 0.3)	-0.3 (-0.6, -0.09)	0.02*

N.B. $FEF_{25-75\%}$, FeNO and absolute BDR associations were adjusted for age, sex, height, relative humidity, temperature, ozone and season of testing. FEV_1/FVC ratio associations were adjusted for relative humidity, temperature, ozone and season of testing. All results are interpreted as per increase in 29 grass pollen grains/m^3. p-int= p-value for interaction term; *p-int ≤ 0.1.

Table 4. **Significant associations** between grass pollen and lung function outcomes; and FeNO on same day, lag 1, lag 2, lag 3 and cumulative 3-day lag in those with current hay fever alone, current hay fever and current asthma, and current asthma alone.

Outcomes	Grass pollen									
	Lag 0		Lag 1		Lag 2		Lag 3		Lag 0-3	
	Coef. (95% CI)	**p-value**	**Coef. (95% CI)**	**p-value**	**Coef. (95% CI)**	**p-value**	**Coef. (95% CI)**	**p-value**	**Coef. (95% CI)**	**p-value**
In those with current hay fever and no asthma (N=44 for spirometry; N=48 for FeNO test)										
$FEF_{25-75\%}$ (ml/second)	-306 (-736, 124)	0.16	-451 (-845, -56)*	0.03*	-508 (-856, -160)*	0.004*	-525 (-907, -142)*	0.007*	-120 (-220, -19)*	0.02*
Absolute BDR (ml)	20 (-40, 80)	0.52	39 (-21, 99)	0.21	63 (-0.003, 125)*	0.05*	63 (-1.5, 128)	0.06	12.5 (-3.8, 29)	0.13
FeNO (ppb)	12.5 (1.5, 23)*	0.025*	16 (4.6, 27)*	0.006*	11.3 (-0.7, 23)	0.066	12.2 (1.0, 23)*	0.03*	3.5 (0.6, 6.1)*	0.02*
In those with current hay fever and current asthma (N=43 for spirometry)										
Absolute BDR (ml)	98 (44, 153)*	<0.001*	95 (44, 146)*	<0.001*	92 (39, 146)*	<0.001*	94 (39, 148)*	0.001*	25 (11, 38)*	<0.001*

In those with current asthma and no current hay fever (N=166 for spirometry)										
Absolute BDR (ml)	56 (-6.4, 119)	0.08	69 (7.8, 131)*	0.03*	63 (7.5, 119)*	0.03*	43.5 (-8.7, 96)	0.10	17.1 (2.6, 32)*	0.02*

N.B. $FEF_{25-75\%}$, absolute BDR and FeNO associations were adjusted for age, sex, height, relative humidity, temperature, ozone and season of testing. All results are interpreted as per increase in 29 grass pollen grains/m^3. *p ≤ 0.05.

Table 5. **Adjusted analysis** of grass pollen and FEV_1; and FeNO stratified by grass pollen sensitisation, with and without current hay fever on same day, lag 1, lag 2, lag 3 and cumulative 3-day lag.

Grass pollen	FEV_1 (ml)				FeNO (ppb)			
	Sensitized to ≥ 1 grass pollen extract without current hay fever n=373		**Sensitized to ≥ 1 grass pollen extract with current hay fever n=426**		**Sensitized to ≥ 1 grass pollen extract without current hay fever n=385**		**Sensitized to ≥ 1 grass pollen extract with current hay fever n=396**	
	Coef. (95% CI)	**p-value**	**Coef. (95% CI)**	**p-value**	**Coef. (95% CI)**	**p-value**	**Coef. (95% CI)**	**p-value**
Lag 0	34 (-53, 122)	0.44	43 (-120, 34)	0.28	1.3 (-3.5, 6.1)	0.60	5.5 (0.09, 11)*	0.047*
Lag 1	53 (-39, 146)	0.26	-37 (-105, 31)	0.28	3.2 (-1.8, 7.8)	0.22	5.2 (-0.09, 11)*	0.05*
Lag 2	57 (-44, 159)	0.27	-67 (-136, 2.3)	0.06	2.9 (-1.9, 7.8)	0.23	5.2 (-0.2, 11)	0.06
Lag 3	30 (-91, 149)	0.63	-78 (-157, -2.5)*	0.04*	2.0 (-1.97, 6.4)	0.31	2.3 (-2.8, 7.8)	0.37
Lag 0-3	13 (-15, 42)	0.35	-16 (-35, 3.8)	0.10	0.6 (-0.6, 2.0)	0.29	1.2 (-0.1, 2.6)	0.07

N.B. FEV_1 and FeNO associations were adjusted for age, sex, height, relative humidity, temperature, ozone and season of testing. All results are interpreted as per increase in 29 grass pollen grains/m^3. *p≤0.05.