The impact of ecosystem nitrogen enrichment on pollen allergy: a cross-sectional paired comparison study

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Summary

Background The prevalence of allergy to aeroallergens is rising, driven by both environmental and lifestyle changes. However, the role of ubiquitous nitrogen enrichment in exacerbating pollen allergy remains unclear. This study aimed to investigate the impact of nitrogen on pollen allergenicity by connecting the resulting ecological changes with allergic outcomes.

Methods We conducted a cross-sectional paired comparison study, examining differences between nitrogen-enriched (fertilised) and non-enriched common semi-natural grasslands in Belgium. Pollen from paired grasslands (n=50, enriched [n=25] *vs* non-enriched [n=25]) based on their common geography, were sampled following a standardised protocol. We analysed grassland pollen abundance, quantified pollen species composition via DNA sequencing, and assessed pollen allergenicity using basophil activation testing and specific IgE measurements in a cross-sectional sample of adults who were allergic to grass pollen (n=20). Basophil activation test outcome measures included area under the dose–response curve, maximal reactivity ($CD63_{max}$), and effective concentration eliciting 50% basophil activation.

Findings Nitrogen-enriched grasslands produced significantly more pollen, with a 6·2-fold increase compared with their unfertilised counterparts (3·6 mg/m² *vs* 0·6 mg/m²). When normalised to protein content, pollen from these enriched grasslands showed increased allergenic potential, with 5·1 times higher basophil activation test sensitivity and a 1·3-fold increase in specific IgE titres compared with their unfertilised counterparts (geometric mean fertilised 3·63 kU_A/L *vs* unfertilised 2·81 kU_A/L).

Interpretation Nitrogen enrichment substantially increased pollen abundance and allergenicity, indicating a heightened allergy burden in nitrogen-rich environments. These findings underscore the need for policies addressing nitrogen pollution to mitigate its public health impacts.

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Introduction

Pollen allergy, encompassing allergic rhinitis, conjunctivitis, and asthma, is a growing public health concern worldwide.[1] It causes a reduced quality of life in patients and has a substantial economic burden due to healthcare costs and lost patient productivity.[2,3] Since the 1980s, the prevalence of pollen allergies has increased globally, partly due to improved diagnostics, but also due to a complex interplay of environmental and lifestyle factors.[4,5]

Among the environmental drivers, nitrogen enrichment has emerged as a substantial potential contributor to the increasing burden of pollen allergies.[6] Sources include the combustion of fossil fuels and the widespread use of synthetic fertilisers in agriculture, which have more than doubled the global amount of biologically reactive nitrogen since the 1950s.[7] Elevated atmospheric oxidised nitrogen compounds, mainly resulting from fossil fuel combustion, have been shown to enhance the allergenic properties of pollen by altering the structure and expression of allergenic proteins.[8,9] Atmospheric nitrogen pollutants can also affect pollen morphology and compromise pollen membrane integrity, leading to an increased release of allergens.[10,11] Additionally, there is evidence that these pollutants can cause epithelial damage and respiratory inflammation in patients who are allergic to pollen, facilitating allergen access and further increasing allergy symptoms and disease severity.[12] Although much attention has been given to the direct effects of increased atmospheric oxidised nitrogen concentrations on respiratory conditions, the potential impact of the ubiquitous ecosystem nitrogen enrichment through agricultural fertilisation and atmospheric deposition on pollen allergy has been largely overlooked.[13]

Because the primary production of many ecosystems is nitrogen-limited, nitrogen enrichment can have far-reaching ecological consequences.[7] Nitrogen enrichment is well recognised to boost primary productivity and favour a few highly competitive plant species, leading to shifts in plant species community composition and

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Research in context

Evidence before this study

The ecological effects of nitrogen enrichment on ecosystems, including increased primary productivity and changes in plant community composition, are well established. However, the subsequent effects on the abundance and composition of airborne pollen are unknown, and the extent to which this might contribute to the severity of pollen allergy in patients who are allergic to pollen has not yet been studied. On Aug 1, 2024, we searched PubMed, Embase, Web of Science Core Collection, and Scopus for literature published between Jan 1, 2001, and July 31, 2024, using combinations of the search terms "nitrogen", "ammonium", "nitrate", "pollen", "pollen allergy", and "allergy burden" among others, with no language restrictions. We found that previous studies addressing the impact of nitrogen pollution on pollen and pollen allergy exclusively focused on the direct effects of oxidised nitrogen pollution on atmospheric pollen levels, pollen structure, allergen structure and release, and allergenic reactivity. The few studies focusing on the effects of increased soil nitrogen availability on pollen were all small-scale experiments and focused on a few individual plant species. They reported increased pollen production and altered biochemical composition of pollen. However, the extent to which increased ecosystem nitrogen availability affects the pollen allergy burden through the combined effects of changes in plant species composition, pollen production, and pollen biochemical composition remains an important knowledge gap.

Added value of this study

Our study shows that nitrogen enrichment of common grassland types not only substantially boosts pollen abundance—by a factor of 6·2 in fertilised versus unfertilised grasslands—but also enhances the intrinsic pollen immunoreactivity, as shown by the 5·1 times higher basophil activation test sensitivity and 1·3-fold increase in specific IgE titres. These findings extend the understanding of nitrogen pollution's impact on public health, revealing that nitrogen enrichment exacerbates pollen allergy severity.

Implications of all the available evidence

The available evidence emphasises the need for updated environmental and public health policies to address the growing issue of pollen allergy as a major public health concern. Together with atmospheric nitrogen pollution, ubiquitous ecosystem nitrogen enrichment can substantially worsen pollen allergies. To mitigate these health impacts, nitrogen emission regulations should not only consider the direct ecological consequences of nitrogen pollution (biodiversity loss) but also its indirect health impacts (eg, allergy disability-adjusted life years). Future research should prioritise evaluating the effects of nitrogen pollution on the disease burden across entire populations on a larger spatial scale, assessing the health-care costs and benefits of various nitrogen reduction scenarios within regional and national policy frameworks. Simultaneously, deepening our mechanistic understanding by further investigating the biochemical mechanisms linking nitrogen enrichment to pollen allergenicity is crucial. By integrating ecological changes with public health outcomes, this study provides crucial guidance for future research and policy actions.

concurrent biodiversity loss.[14] Grasslands, which are among the most nitrogen-sensitive ecosystems, typically experience a loss of forb species and an increased dominance of nitrophilic grasses.[15,16] This shift not only alters the structure and functioning of these ecosystems, but might also have implications for the quantity and composition of airborne pollen.[13,17] Grasses, in particular, are major contributors to pollen-related allergic diseases, and the proliferation of specific species can be expected to exacerbate the severity of the pollen allergy season.[18–20]

Although shifts in primary productivity and plant community composition have been hypothesised to influence pollen abundance and composition, this relationship has not yet been explored in the context of ecosystem nitrogen enrichment. Furthermore, the potential downstream effects of such changes on pollen-related allergies remain unquantified.[13,21] While a few studies have documented increased pollen production through experimental nitrogen fertilisation in some plant species of agronomic importance, such as *Cucurbita*, *Juniperus*, *Malus*, and *Festuca*, no research so far has assessed the broader implications of nitrogen-induced changes on pollen allergy within a real ecosystem setting.[22–25] In addition to altered pollen production and composition, nitrogen enrichment has also been hypothesised to lead to changes in pollen protein content and biochemical properties, which could further increase the immune response in sensitised individuals (ie, those who show IgE-mediated immune reactivity towards pollen samples), increasing the overall burden of pollen allergy.[6]

In an era of ever-increasing nitrogen enrichment worldwide, particularly in densely populated urban and industrial areas, and regions with intensive agriculture, understanding the extent to which these increases might influence the prevalence and severity of pollen allergy is crucial.[7] Regarding this critical knowledge gap, our study aimed to address the impact of nitrogen enrichment on pollen allergenicity. We used a combination of an ecosystem field study and subsequent clinical analyses, thus providing a comprehensive assessment of the pathways through which nitrogen enrichment might affect pollen allergy. Our findings provide crucial insights for both environmental policy and public health,

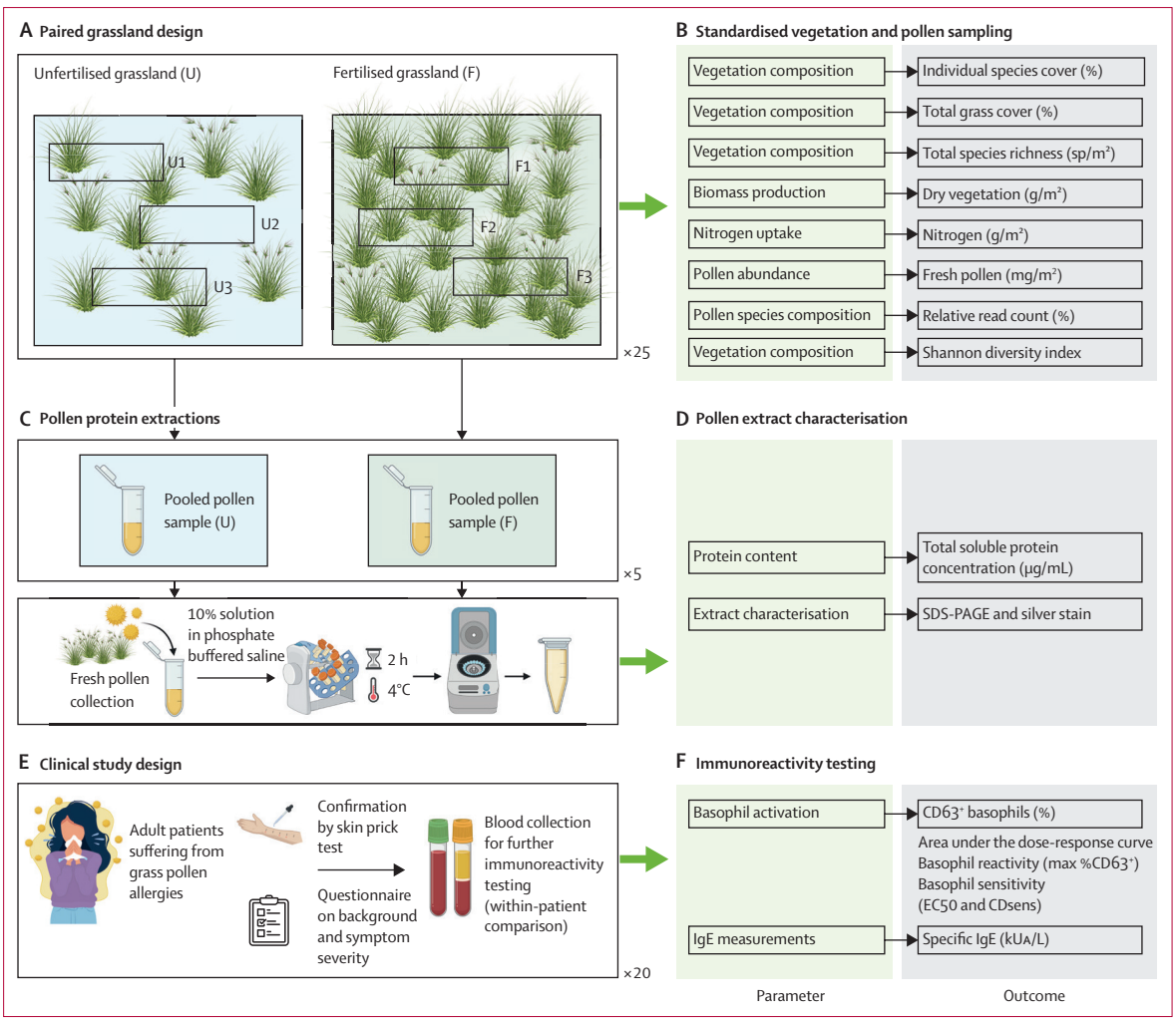

Figure 1: **Schematic presentation of the study design**
Paired pollen sampling design. U1-U2-U3 and F1-F2-F3 represent the three transects in each unfertilised or fertilised grassland (A). Different parameters derived from each sampled transect and their outcome variable (B). A subset of pollen samples (n=10) was processed for inclusion in the clinical study. Pollen samples from all transects were pooled per grassland and a standardised extraction protocol was used to make total soluble protein extracts (C). Protein concentration was used for sample normalisation and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis for extract characterisation (D). Clinical study design (E). Derived immunoreactivity parameters on extracts normalised for protein content (F).

particularly in the context of mitigating the adverse health impacts of nitrogen pollution.

Methods

Study design and standardised pollen sampling

We used a paired design at 25 grassland sites within the biogeographical region of Hageland, Belgium, each pair consisting of an unfertilised semi-natural grassland and an adjacent fertilised grassland, including Fen Meadows (n=18), Grass Heaths (n=16), and Hay Meadows (n=16; figure 1; appendix p 4). The average distance between grassland pairs was 5839 m (SD 3259) and the average distance between grasslands within a pair was 31 m (28). All pairs were formerly part of the same pristine grassland ecosystem, but since the 1980s, one grassland of each pair came under agricultural management and was annually fertilised at rates of 50 up to 350 kg N ha^{-1} $year^{-1}$. The paired design isolates the effects of nitrogen fertilisation, controlling for confounding factors such as potential other site differences like soil characteristics or the presence of air pollutants that can directly alter pollen-allergenic properties. The selected grassland ecosystems were Fen Meadows (wet nutrient-poor grasslands), Grass Heaths (dry-to-moist nutrient-poor grasslands), and Hay Meadows (dry-to-moist naturally nutrient-rich grasslands). These were among the most common semi-natural grassland types in western Europe, but have substantially declined since the 1950s largely due to agricultural conversion and are now protected under the European Habitats Directive.[26] Although unfertilised semi-natural grasslands have become increasingly rare, these three types remain

See Online for appendix

among the most prevalent in our study region and by extension, in western Europe (appendix pp 5, 19). The fertilised grasslands we selected adjacent to these sites are typical production grasslands with a more uniform vegetation composition and are representative of the majority of grasslands in Belgium (appendix pp 5, 19).

In both the unfertilised and fertilised part of each grassland site, pollen was collected along three 2 m×20 m transects with a handheld Dyson V6 vacuum-pump with two mounted VWR stainless steel sieves with apertures of 75 µm and 25 µm to filter out debris and retain pollen. To ensure a standardised measurement of grassland pollen abundance, we used a protocol in which each transect was sampled for exactly 2 min, moving through the vegetation at different heights to capture all potential pollen-producing plants. Two sampling rounds were carried out at the peak of the grass pollen season, more specifically during the second and fourth week of May 2023. Subsequently, the relative plant cover (%) of all plant species along the transect was assessed using three 1 m×1 m quadrats. Three vegetation samples (0·2 m×0·2 m, cut at ground level) were taken from each transect and dried for 72 h at 60°C to quantify total biomass production (ton/ha). Total nitrogen concentration of the vegetation (%) was determined by elemental analysis (EA1108 Elemental Analyzer, CARLO ERBA Reagents, Italy) and multiplied with the biomass production to calculate total nitrogen uptake (g/m²). This measure provides an accurate estimation of the plant's available nitrogen in the soil, to confirm higher nitrogen availability in fertilised versus unfertilised grasslands.

Fresh pollen samples (n=300) were immediately transferred to the lab, weighed to quantify grassland pollen abundance (mg/m²), and stored at –80°C for later use. 236 samples (123 from fertilised and 113 from unfertilised grasslands) contained sufficient pollen (>1 mg) to allow for genomic DNA extraction using the Plant/Fungi 96-well plate DNA Isolation Kit (Norgen Biotek Corp, Thorold, ON, Canada), followed by DNA metabarcoding at GenomicsCore, UZ Leuven (appendix p 2). The high-throughput sequencing data were processed to identify all plant taxa in each pollen sample using the metabarcoding pipeline developed by Keller[27] (appendix p 2). Additionally, pollen samples from ten grasslands (five pairs) were used to quantify pollen immunoreactivity (figure 1). Therefore, pollen from each of the three transects within each grassland sampled in round one were pooled. This selection was based on sample volume and purity, ensuring a manageable sample size for the clinical analyses. Our selection ensured inclusion of all three grassland types, each with a representative vegetation composition for the respective type.

Extraction and quantification of soluble proteins

The selected pollen samples (n=10) were suspended in Dulbecco's phosphate buffered saline (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) at pH 7 to create a 10% (weight in volume) solution. Total soluble proteins were extracted by continuous stirring for 2 h at 4°C. The suspension was centrifuged at 4663 g for 10 min at 4°C. The supernatant was collected, centrifuged again at 21130 g for 20 min at 4°C, and filtered through a 0·22 µm antimicrobial filter. The total soluble protein content of the pollen extracts was quantified using the Bradford assay (B6916, Sigma-Aldrich, Bradford; appendix p 2). Protein concentration was used for normalisation in further experiments, and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis was used for extract characterisation (appendix pp 2, 11). Aliquots were stored at –80°C until further use.

Clinical study design and patient inclusion

A cross-sectional paired comparison study (within-subject design) was done with adult patients (ie, those aged >18 years) suffering from grass pollen allergies who had not received desensitisation treatment within 2 years before inclusion. The study followed the STROBE guidelines for cross-sectional studies.[28] Ethical approval was granted by the Ethical Committee Research of UZ/KU Leuven (S65184), and informed consent was obtained from all patients. Patient recruitment took place outside the grass pollen season, from Jan 17, to April 2, 2024, during routine consultations at the Allergy Department in the University Hospitals Leuven and via a flyer campaign targeting volunteers visiting the university campus or the Sciensano AirAllergy website. Allergies were confirmed through routine skin prick testing for grass pollen (appendix p 21), with a positive test defined as a reaction exceeding the histamine control. All patients completed a questionnaire on their socio-demographic background and symptom severity (table; appendix p 20). Whole blood samples were collected for basophil activation testing and serum for immunoreactivity testing. Each patient served as their own internal control, as we compared reactivity toward pollen from unfertilised versus fertilised grasslands, normalised for protein content, in each patient. Allergen-specific IgE levels for Timothy grass pollen (g6, Phadia Thermo Fisher Scientific) were measured by the routine clinical laboratory of UZ Leuven.

Immunoreactivity measurements

To measure the allergenicity of our pollen samples, two established in-vitro immunoreactivity tests were used to measure basophil activation and specific IgE levels in human blood samples. The basophil activation testing was done following a previously described standardised in-house protocol (appendix p 3). Fresh blood samples were stimulated with different concentrations of pollen extracts (ie, 1 µg/mL, 100 ng/mL, 10 ng/mL, 1 ng/mL, 0·1 ng/mL) for 20 min at 37°C. Cells were stained with anti-CD123, anti-HLA-DR, and anti-CD63 (BioLegend, San Diego, CA,

USA). Basophils were gated as $CD123^+$ and $HLA\text{-}DR^-$ cells and CD63 expression was used as a measure for basophil degranulation or activation. Basophil activation test outcome parameters analysed included area under the dose–response curve, basophil reactivity (max $\%CD63^+$ basophils), and basophil sensitivity evaluated as effective concentration eliciting 50% basophil activation (EC50) and CDsens ([1/EC50]*100). Specific IgE towards our pollen samples was measured using the ImmunoCAP assay. Prewashed Streptavidin ImmunoCAPs (Ro212, Phadia Thermo Fisher Scientific, Uppsala, Sweden) were loaded with 2·5 µg biotinylated pollen protein extracts (appendix p 3), incubated for 30 min, washed, and used for specific IgE measurements in patient sera using a Phadia UniCAP100 system (Pharmacia Diagnostics AB, Uppsala, Sweden). The commercial ImmunoCAP for Timothy grass pollen (g6, Phadia) served as a positive control (appendix p 16).

Statistical analysis

The effects of fertilisation on vegetation characteristics and pollen abundance were analysed in R (v.4.3.1).[29] All statistical models were constructed with fertilisation status, grassland type, and their interaction as fixed factors, and site as a random factor to account for the non-independence of grassland pairs. Differences in plant composition between pairs were quantified using redundancy analysis on Hellinger-transformed plant cover data.[30] Significance of axes and variables in the final model was tested using permutations (n=999), and pairwise differences between fertilised and unfertilised grasslands were calculated using Bray–Curtis distances. Next, a generalised linear mixed model was created with plant species richness as the response variable, using a Poisson distribution with a log link function. A generalised linear mixed model with a beta distribution and logit link function was fitted for the grass cover. No overdispersion was detected in the models. Additionally, linear mixed models were constructed to analyse biomass production, nitrogen uptake, and pollen abundance. Response variables were log-transformed to meet model assumptions of normality and heteroscedasticity. Estimated marginal means and 95% CIs were calculated to visualise differences between fertilised and unfertilised grasslands, with pairwise differences determined using Tukey's Honest Significant Difference post-hoc tests. For comparing the species composition of pollen samples, the same method as for vegetation composition was applied to the Hellinger-transformed pollen sequencing read counts.

Next, the protein content and immunoreactivity measures of the selected pollen samples were compared between fertilised and unfertilised grasslands using a within-patient comparison design. This design entails testing pollen samples from paired fertilised and unfertilised grasslands (n=10) within each of the patients (n=20), enhancing the study's power to detect meaningful differences between paired samples. The patient sample size was calculated based on the first three included patients to achieve 80% power at a significance level of 0·05, resulting in a sample size of 11 patients, which was rounded up to 20 for increased robustness. The normality of the data was evaluated using the Shapiro–Wilk test. Comparisons between paired pollen samples were made with the Wilcoxon matched-pairs signed rank test. Statistical analysis was performed using GraphPad Prism 10.1.2 (GraphPad Software).

	n=20
Sex	
Female	13 (65%)
Male	7 (35%)
Age, years	
Median (IQR)	39·5 (31·8 to 50·8)
Range	19–62
BMI	
Weight, kg	68·6 (14·8)
Length, cm	170·7 (11·0)
BMI, kg/m²	23·4 (3·5)
Smoking	
Yes	0
No	20 (100%)
Hay fever symptoms	
Yes	20 (100%)
No	0
Seasonality symptoms	
Spring (March, April, and May)	16 (80%)
Summer (June, July, and August)	16 (80%)
Autumn (September, October, and November)	7 (35%)
Winter (December, January, and February)	7 (35%)
All year long	4 (20%)
Mono-sensitised or polysensitised	
Mono-allergic to grass pollen	1 (5%)
Polysensitised	19 (95%)
Polysensitised to grass pollen and herbaceous plants	12 (60%)
Specific IgE, kU_A/L	
Birch pollen, mean (range)	15·1 (0·34 to >100·0)
Timothy grass pollen, mean (range)	2·32 (<0·10 to 28·8)
Disease severity (total nasal symptom score)	
Last 12 h	2·85 (3·00)
Last 2 weeks	3·50 (3·27)
Peak of symptoms last 12 months	7·25 (2·57)
Disease severity (visual analogue scale)	
Average score last 3 months	36·79 (16·81)
Average score at peak of symptoms last 12 months	49·67 (16·60)

Data are n (%) or mean (SD) unless otherwise stated. Inclusion period from Jan 17, 2024, to April 2, 2024.

Table: **Socio-demographic characteristics of the study population**

Role of the funding source
The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results
Redundancy analysis showed that paired unfertilised and fertilised grasslands had a significantly different vegetation composition (figure 2). Grasses (Poaceae) dominated the fertilised grasslands with a mean cover of 61% (95% CI 51–70), nearly twice the mean grass cover in the unfertilised grasslands (32% [24–40]; figure 2). Additionally, fertilised grasslands had a lower mean plant species richness than unfertilised grasslands (10·0 species/m² compared with 13·7 species/m²). The total biomass production and nitrogen uptake were on average 71% and 77% higher, respectively, in the fertilised grasslands compared with the unfertilised grasslands (figure 2). Standardised pollen sampling showed a mean pollen abundance of 3·6 mg/m² for the fertilised grasslands, 6·2 times higher than the unfertilised grasslands (0·6 mg/m²). Additionally, the plant species composition of the pollen samples significantly differed between paired fertilised and unfertilised grasslands (figure 2). DNA metabarcoding of the pollen samples indicated that in fertilised grasslands *Ranunculus* sp had on average the highest relative read count (37%), followed by *Alopecurus pratensis* (16%), and *Plantago lanceolata* (16%; appendix pp 6–7). In unfertilised grasslands, *Ranunculus sp* also had the highest relative read count (40%), followed by *Plantago lanceolata* (26%) and *Alopecurus pratensis* (7%). Significant differences between paired unfertilised and fertilised grasslands were also found for each grassland type separately, except for Grass Heaths for which plant species richness and pollen abundance did not significantly differ (appendix pp 8–9).

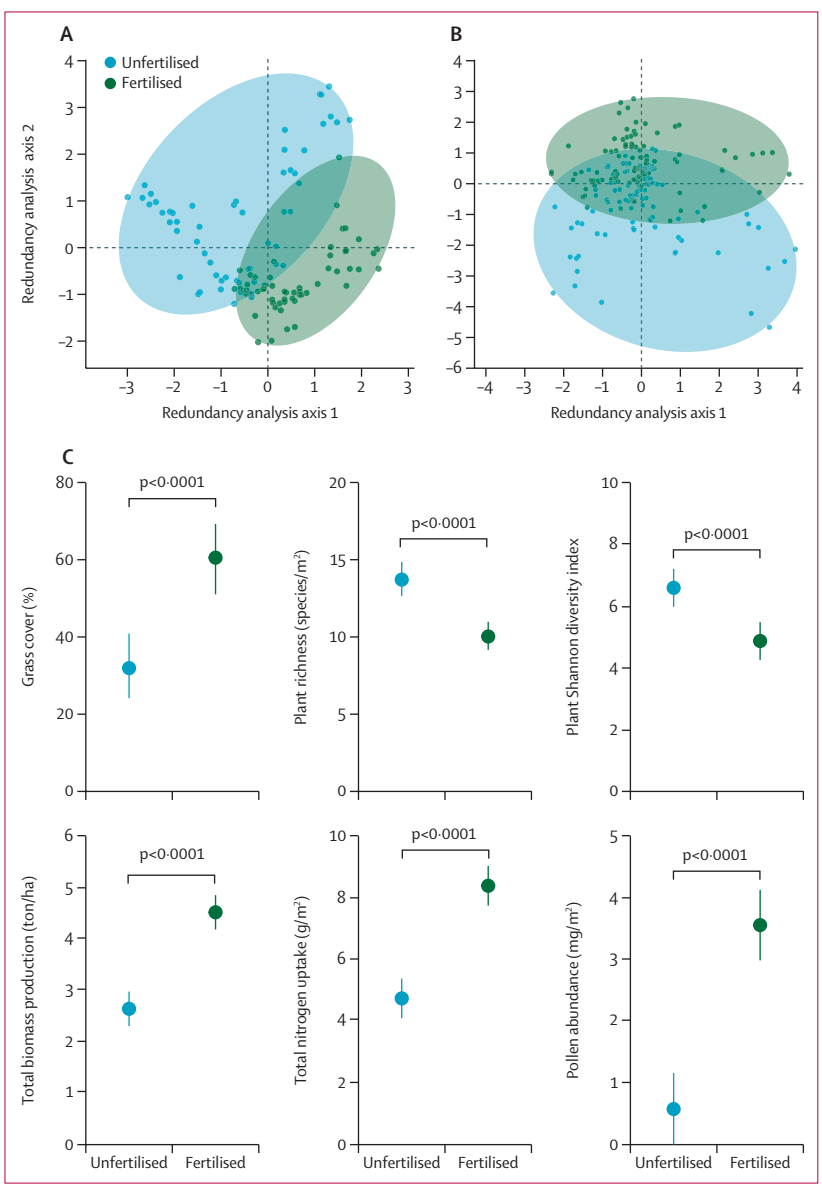

Figure 2: **Grassland vegetation and pollen characteristics**
Redundancy analysis biplots visualising the plant species composition of the grasslands (A) and pollen samples (B). Points illustrate individual vegetation surveys and pollen samples, respectively. Points that are closer together are characterised by a more similar plant species composition. Colours and ellipses depict the fertilisation status of the grasslands from which samples were collected. Estimated marginal means with error bars showing 95% CIs (C).

For the clinical study, 31 patients with grass pollen allergy were included, of whom a final subset of 20 patients was eligible for further testing (appendix p 10). One patient, with negative grass pollen skin prick testing, was included as a negative control. 13 (65%) patients were female and seven (35%) were male, the median age was 39·5 years (IQR 31·8 to 50·8), and the mean BMI was 23·4 kg/m² (SD 3·5; table). All patients experienced hay fever symptoms, 16 (80%) patients both during spring and summer. One (5%) patient was monosensitised to grass pollen, while 19 (95%) were polysensitised, of whom 12 (60%) were polysensitised to pollen from both grasses and herbaceous plants. Routine ImmunoCAP measurements showed mean specific IgE values of 2·32 kU_A/L (range <0·10 to 28·8) for Timothy grass pollen.

For basophil activation testing, all patients showed a dose-dependent reactivity. The lowest dose elicited responses ranging from 0% to 91%, while the highest dose elicited responses from 5% to 99%. For the three intermediate concentrations, a stronger reactivity toward pollen from nitrogen-enriched grasslands was observed (figure 3). Basophil activation test reactivity and sensitivity parameters of these patients toward pollen from fertilised grasslands were higher compared with unfertilised grasslands (area under the dose–response curve mean unfertilised 47 200 %CD63⁺×ng/mL and mean fertilised 53 235 %CD63⁺×ng/mL; max %CD63⁺ mean unfertilised 78·2 and mean fertilised 83·3; EC50 mean unfertilised 150·3 and mean fertilised 38·8; and CDsens mean unfertilised 53·7 and mean fertilised

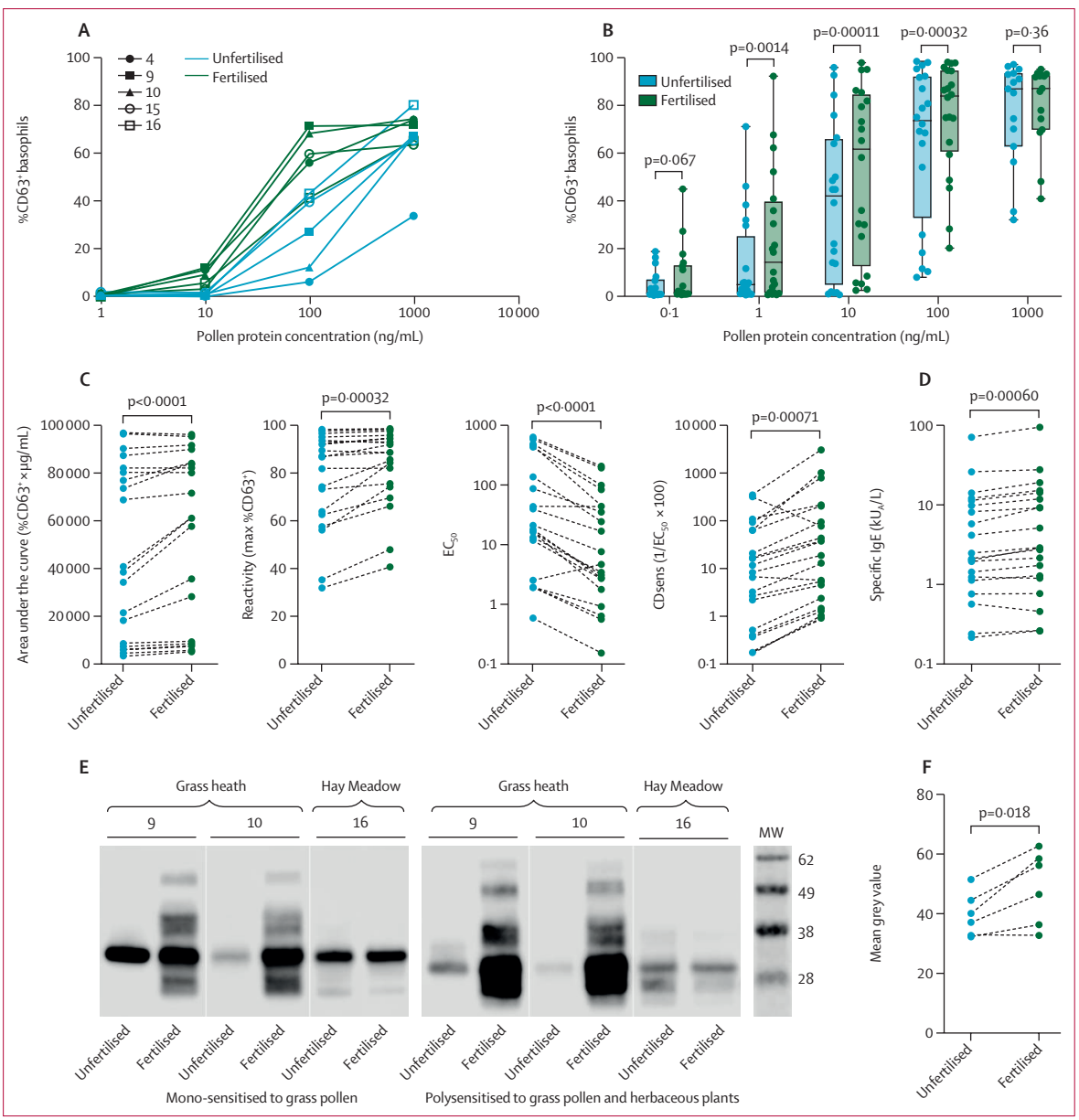

***Figure 3*: Immunoreactivity of grassland pollen samples**
Exemplary dose–response basophil activation of all pollen samples tested on one patient included in the study (A) and basophil activation of all included patients. Data are shown with boxplots showing median, IQR, and whiskers extending to the minimum and maximum values (B). Area under the dose–response curve, basophil reactivity (max %CD63$^+$), EC50, and CDsens (1 / EC50 * 100), between the unfertilised and fertilised group (C). Specific IgE values in the unfertilised group and fertilised group (log_{10}-transformed scale of the y-axis; paired *t* test of log_{10}-transformed data; D). Comparison of allergenicity of two selected patients sensitised to grass pollen via immunoblot (E). Immunoblot quantification shows mean grey values. The graph shows the mean value of two (n=4) or three (n=2) pollen extracts. The differences between unfertilised and fertilised groups were compared via a paired *t* test. The dotted lines represent coupled measurements per patient. The Wilcoxon matched pairs signed rank test was used to determine significance unless stated otherwise (F). EC50=effective concentration 50% activation.

275·3; figure 3; appendix p 12). We observed a 1·1-fold increase in area under the dose–response curve, a 3·9-fold decrease in EC50, and a 5·1-fold increase in CDsens, showing higher basophil sensitivity in fertilised grassland compared with unfertilised grassland.

For specific IgE, 1·3-fold higher values were observed towards fertilised versus unfertilised pollen (geometric mean unfertilised 2·81 kU_A/L and fertilised 3·63 kU_A/L; figure 3). Because of the limited availability of two of the ten pollen extracts (number 4 and number 10), two unfertilised samples were only tested in 10 and 15 patients, respectively. Immunoblot analysis revealed that patients exhibited a stronger response to specific allergens and reacted to a broader range of proteins in the fertilised compared with the unfertilised pollen samples (figure 3; appendix p 15).

Comparison of the three different types of grasslands separately showed that the difference in basophil sensitivity and specific IgE measurements was driven by Fen Meadows and Grass Heaths (appendix pp 13–14).

Discussion

To the best of our knowledge, this study is the first to establish a clear relationship between ecosystem nitrogen enrichment and pollen allergenicity. Our findings show that agriculturally fertilised grasslands have a higher pollen abundance than their unfertilised semi-natural counterparts. Moreover, this pollen exhibited significantly higher intrinsic allergenic potential, as evidenced by increased basophil sensitivity and elevated specific IgE levels in patients who were allergic to grass pollen.

Previous research has established that nitrogen enrichment impacts grassland ecosystems by increasing biomass production, reducing biodiversity, and altering plant community composition, prompting strict environmental policy to mitigate these adverse effects.[14,26] Similarly, we also found that fertilised grasslands exhibit higher productivity, plant species loss, and a shift towards grass-dominated vegetation. However, we go beyond these adverse ecological changes by linking them to human health outcomes, specifically the exacerbation of pollen allergies.

Firstly, we show a 6-fold increase in pollen abundance in nitrogen-enriched grasslands, suggesting that nitrogen-enriched environments most likely present a more potent allergenic landscape than non-nitrogen-enriched environments. Although earlier studies have reported increased pollen abundance in individual species under higher soil nitrogen availability (eg, *Festuca rubra*), our study is the first to quantify this at an ecosystem level, highlighting the broader implications of nitrogen pollution for allergenic pollen loads.[25] Higher pollen abundance in fertilised grasslands could result from increased biomass production, either through enhanced growth of individual plants or a higher plant density. Additionally, the increased presence of species known for high pollen production, such as grasses (eg, *Alopecurus pratensis*, which consistently showed greater cover in fertilised grasslands across all types), could contribute to the observed pattern. Understanding the relative contribution of these mechanisms to the overall increase in pollen abundance in fertilised grasslands calls for a more detailed investigation at the species level within these ecosystems.

Secondly, our analyses also showed a higher intrinsic allergenic potential for pollen samples from fertilised grasslands, shown by an increased sensitivity (CDsens) in basophil activation testing by a factor of 5·1. This might be explained either by changes in the plant species composition of the pollen, leading to the presence of more allergenic grass species or other cross-reactive plant species, by altered biochemical properties of the pollen, or by a combination of both.[7,14] For example, pollen samples from fertilised grasslands had a higher abundance of *Alopecurus pratensis*, known to contain major allergens such as group 1 and group 5 allergens.[31] Notably, the clinical study assessing the allergenic potential was normalised to protein content, indicating that the observed increase in allergenic potential is independent of the concurrent rise in pollen abundance. To identify whether the increased presence of pollen of a specific plant species in nitrogen-enriched grasslands would contribute to the observed heightened reactivity, or if nitrogen-induced altered biochemical properties of pollen (irrespective of plant species) are responsible, further plant species-specific testing is required. Additionally, to address true clinical patient reactivity, direct clinical testing such as nasal allergen provocation testing and pollen chamber exposure would be necessary.

Furthermore, a detailed analysis of the three different grassland types showed that the significant increase in ex-vivo pollen allergenicity was mostly shown in Fen Meadows and Grass Heaths. Pristine examples of these grassland types are characteristically very nutrient-poor, particularly compared with naturally more nutrient-rich Hay Meadows.[26] Yet, we also observed significantly higher ex-vivo allergenicity in pollen from fertilised Hay Meadows, indicating that, even in already nutrient-rich grasslands, additional nitrogen enrichment still leads to elevated ex-vivo allergenicity. Moreover, although the already nutrient-rich Hay Meadows expectedly exhibited the smallest relative increase in nitrogen uptake between unfertilised and fertilised grasslands (1·6-fold increase), they markedly showed the highest increase in total pollen abundance (9·3-fold increase).

Notably, all grasslands within the study region are already under the influence of nitrogen enrichment, even without direct agricultural fertilisation that could range between 50 kg N ha^{-1} $year^{-1}$ and 385 kg N ha^{-1} $year^{-1}$. Indeed, in Belgium, the extant atmospheric nitrogen deposition is among the highest globally, frequently above 20 kg N ha^{-1} $year^{-1}$. These levels exceed critical loads of 5–10 kg N ha^{-1} $year^{-1}$ of environmental policy to prevent biodiversity loss in (unfertilised) semi-natural grasslands.[15,26] Therefore, a similar paired analysis done on pollen from grasslands in regions with low atmospheric nitrogen deposition would probably reveal even greater differences in pollen allergen production and pollen allergenic potential. Therefore, our study likely underestimates the true impact of nitrogen pollution on pollen allergenicity, either via direct fertilisation or indirectly via atmospheric nitrogen deposition. Furthermore, although our selected semi-natural grassland types constituted the most common European grassland types before the advent of artificial nitrogen fertilisers, this type of grassland has declined

dramatically since the 1950s in favour of grassland under intensive agriculture, granting them strict protection under the European Habitats Directive.[26] In the investigated region alone, the surface area of these semi-natural grasslands dropped by approximately 92%. Therefore, even though our study provides a highly novel insight into the effects of nitrogen enrichment on ex-vivo pollen allergenicity, further research on a larger spatial scale and also in regions with low levels of atmospheric nitrogen deposition remains essential to more accurately estimate the overall allergy burden associated with nitrogen pollution. Given the drastically altered surface areas of semi-natural grasslands in favour of agriculturally improved grasslands in the past decades, investigating how these changes could have contributed to the observed increase in pollen allergy in the past decades is also merited.[26]

In conclusion, our study is, to the best of our knowledge, the first to show that ecosystem nitrogen enrichment can significantly increase pollen abundance and pollen allergenicity. Strengths of this study include the paired design, within-patient comparison, and use of widely accepted ex-vivo functional tests to assess allergenicity. Our results suggest a multiplicative effect for patients who are allergic to grass pollen, potentially leading to a substantial increase in the associated disease burden. In an era of ever-increasing nitrogen pollution worldwide, particularly in regions with intensive agricultural practices, our findings highlight the urgent need for integrated strategies to not only address its biodiversity and environmental impact but also the human health impact posed by nitrogen pollution.[7]

Contributors

RD, PV, OH, TC, RA, and RS conceptualised the study. OH, TC, RA, and RS acquired funding. RD, PV, and RA contributed to project administration. RD, PV, TC, and RS contributed to the method. RD, PV, TR, SK, GP, LC, and TC contributed to the study investigation. RD, PV, TR, SK, AD, and TC contributed to data curation. RD and PV formally analysed the data. RD and PV contributed to data visualisation. LVG, NB, OH, TC, RA, and RS supervised the project. RD and PV wrote the original draft. RD, PV, TR, and SK have assessed and validated the underlying data reported in the manuscript. All other authors had access to the data upon request. GF provided us with resources. PV, SK, LVG, and RS contributed to the inclusion of patients in the study. All authors read and reviewed the manuscript and had final responsibility for the decision to submit it for publication.

Declaration of interests

We declare no competing interests.

Data sharing

The de-identified data used in our analyses will be made available upon reasonable request from 30 days after the finish date of the NITROPOL project. The data can be obtained from the corresponding author. Data will be shared after the approval of a proposal with a signed data access agreement.

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